THE EFFECT OF ANTIFREEZE PROTEINS ON THE COLD TOLERANCE OF GOLDFISH (Carassius auratus L.)

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The Effect Of Antifreeze Proteins On The Cold Tolerance Of Goldfish (*Carassius auratus* L.)

by
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Abstract

The antifreeze proteins (AFP) and glycoproteins (AFGP) of high latitude marine teleosts are an adaptation to extreme low temperature in the presence of ice. Through their affinity to ice crystals, antifreeze proteins are able to adsorb to the surface of ice crystals and prevent their growth. In this way, fish producing them are able to live at temperatures one or more degrees below their plasma freezing point without freezing.

Recent studies point to the possibility that antifreeze proteins may play a role in the nonfreezing cold adaptation of the fish producing them. To test the ability of antifreeze proteins to increase the cold tolerance of fish, warm-acclimated goldfish were injected with antifreeze proteins and exposed to various nonfreezing low temperatures. All three antifreeze types tested (Type I AFP from winter flounder, Type III AFP from ocean pout, and AFGP from Atlantic cod) increased the cold tolerance of goldfish as evidenced by the proportion of antifreeze-injected fish recovering from cold narcosis or cold coma. The AF(G)Ps did not increase the amount of time goldfish could remain exposed to a test temperature before the onset of cold coma, nor did they decrease the temperature at which narcosis occurred when temperature was lowered gradually. These results suggest that antifreeze proteins prevent or reduce cell damage arising from cold shock at rapidly reduced temperatures. The results of this study, along with those of previous studies, suggest a role for antifreeze proteins in nonfreezing cold tolerance and have implications for cold water aquaculture.

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Introduction

I. Effects of Cold Temperature

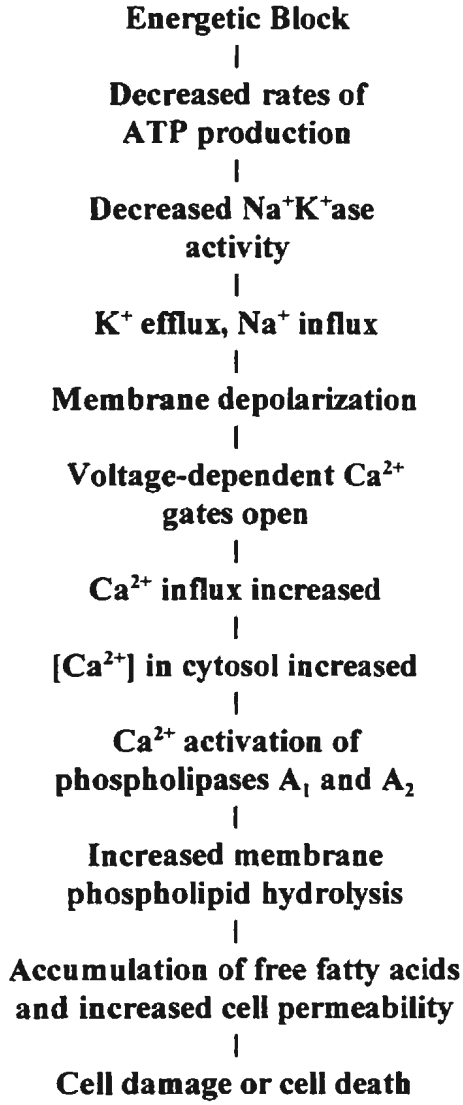
Temperature is a life-limiting environmental factor governing rate of development and growth, and setting limits of metabolic rate and therefore activity. Temperature can have a direct effect on the structure of enzyme proteins and can influence the rate of enzyme-catalysed reactions. According to Van't Hoff's rule, the rate of a chemical reaction approximately doubles for each 10°C increase in temperature. With a reduction in temperature the proportion of substrate molecules with sufficient energy to react decreases and therefore the reaction rate decreases (Clarke, 1987, 1991).

Some of the observed effects of temperature on enzyme systems may be the result of temperature effects on membranes. For proper functioning, membrane lipids must be in a fluid-crystalline state in which the alkyl groups have rotational freedom and the lipids are capable of two-dimensional diffusion in the plane of the membrane (Franks, 1985). As temperature decreases, phospholipid bilayers undergo a reversible phase transition from a liquid-crystalline state to a gel state . The temperature of this phase transition depends on the chain length and degree of saturation of acyl chains (Franks, 1985), and for biological membranes the phase transition temperature ranges from 10 - 30°C (Alberts *et al.*, 1989). In the gel phase, membrane lipids become ordered and hydrocarbon residues adopt a fully extended trans conformation making them rigid with decreased rotational freedom is

lost (Rawn, 1989). Phase changes lead to decreased rates of membrane processes and can also result in membrane damage and leakage.

Cold sensitive animals face two major problems at hypothermic temperatures. One problem is the inability to maintain a regulated metabolism. Metabolic rate is depressed at low temperatures as is the regulation of metabolism due to the thermodynamic effects of temperature on enzyme systems, and to substrate limitation. Another problem is the maintenance of ion gradients. Temperature has a differential effect on passive or diffusion-based ion fluxes and active or metabolically-linked ion fluxes (Hochachka, 1986). In cold-sensitive cells, the decoupling of active and passive ion transport leads to what Hochachka calls a programmed cell death (Fig.1). Decreased temperature leads to decreased ATP production due to the effects of temperature on active transport. Passive ion transport is not affected and this leads to membrane depolarization. Calcium influx increases and phospholipases become activated leading to membrane lipid hydrolysis, cell damage, or cell death. In order for cold-sensitive cells to become cold-tolerant they must be able to balance active and passive ion flux. This balance can be achieved in one of two ways, by increasing ion pump capacity or by decreasing membrane permeability (Hochachka, 1987).

Fig.1. Programmed cell death in cold-sensitive cells exposed to cold temperatures. Adapted from Hochachka (1986).



II. Adaptations to a Cold Environment

Animals that are exposed to cold temperatures, either seasonally or continually, survive due to the evolution of some degree of compensation in their physiological processes, and many strategies to counteract the effects of low temperature on enzyme systems can be found. For example, if the amount of active enzyme present at low temperatures is increased, this will result in an increase in the rate of a reaction for a given substrate concentration. This is the situation found in goldfish muscle. The amount of cytochrome oxidase in the skeletal muscle of goldfish acclimated to a temperature of 5°C is 66% greater than in those acclimated to 25°C (Clarke, 1987). A problem arises with this type of compensation in that there is a limit to the solvent capacity of the cell and an increase in the amount of active enzyme may involve a time delay of hours, days, or weeks.

A more common strategy to combat cold is the production of isozymes, enzyme variants that are better able to function at low temperatures. Rainbow trout (*Oncorhynchus mykiss)* produce two functionally different forms of brain acetylcholinesterase, one at low temperatures (2°C) and one at high temperatures (17°C). This is a necessary adaptation since rainbow trout experience seasonal temperature variations from just above zero to almost 20°C (Clarke, 1987).

The need to maintain membrane fluidity at low temperatures has led to alterations in membrane structure known as homeoviscous adaptation, the chemical composition of membranes is regulated to maintain membrane fluidity and protect the functional properties

of the membrane (Cossins and Raynard, 1987). Underlying the changes in membrane structure is a change in lipid composition such as fatty acid composition, phospholipid head-group composition, phospholipid to cholesterol ratios, position of fatty acids on the phospho-glycerol backbone, and the type of linkage between the fatty acyl chain and the phospho-glycerol backbone (Clarke, 1983). Fluidity of membranes can be increased by an increase in fatty acyl chain length, and the insertion of double bonds into the acyl chain (Alberts *et al.*, 1989).

Ectotherms living in cold regions, and those acclimated to cold temperatures in the laboratory, have a higher proportion of unsaturated fatty acids in their membranes than those living in warmer regions or acclimated to higher temperatures. These unsaturated fatty acids have a lower melting point than their saturated homologues, occupy greater areas of the membrane, and are individually more fluid. Adaptive changes to lipid membranes are probably achieved through the regulation of the composition of the fatty acyl-coenzyme-A pool by dietary fat intake and *de novo* synthesis of fats (Cossins and Raynard, 1987).

III. Adaptations to a Freezing Environment

Organisms living in very cold regions can be presented with the additional problem of the need to avoid or tolerate the freezing of their body fluids. Fresh water teleosts are hyper osmotic to their environment and have freezing points of -0.5 to -0.8°C. Since the freezing point of fresh water is about 0°C, these fish are not normally faced with the problem

of freezing and must instead contend with reduced oxygen and food availability as a result of surface ice cover (Marchand, 1989).

The body fluids of most marine teleosts are hypo osmotic to seawater. They contain about one third as much salt as seawater and therefore have a freezing point between -0.7 and -0.9°C. During the winter, temperatures in the polar and coastal north temperate oceans can fall to between -1.7 and -1.9°C, and therefore fishes living in these waters may be supercooled by about 1°C (DeVries, 1983). Supercooling (lowering of body fluid temperature below its equilibrium freezing point) in the presence of ice can be deadly due to the likelihood of nucleation and flash freezing. At present, the only vertebrates known to tolerate the freezing of their body fluids are some terrestrially hibernating amphibians and reptiles (Storey, 1990). Thus, animals living in ice-laden polar and north temperate oceans have evolved the following strategies to avoid freezing.

1) Avoidance of contact with ice through habitat selection. Surface ice crystals can be hard to avoid in shallow seawater, and therefore some shallow water fish make winter migrations into deeper water where they can avoid ice crystal contact (DeVries, 1983). Other species such as the cunner (*Tautogolabus adspersus)* avoid ice contact by overwintering in a torpid state in protected microhabitats such as caves and crevices (Valerio *et al.*, 1990).

2) Production of compounds, such as reducing sugars, that stabilize the supercooled state. Supersaturated solutions of most polyols and sugars are stable at low temperatures for long periods of time. Polyols stabilize the native state of proteins and inhibit

thermal denaturation. They depress the freezing and supercooling points colligatively. Examples of supercooling stabilizers include glycerol, sorbitol, mannitol, and ethylene glycol. The killifish (*Fundulus heteroclitus*) increases its blood glucose level at subzero temperatures. The hydroxyl-rich glucose seems to act as a supercooling stabilizer by preventing spontaneous nucleation in the absence of ice crystals (Theede, 1973).

3) Development of strategies to avoid freezing in the presence of ice. If a fish can adapt in a way that allows it to remain in icy waters, it can exploit a habitat which is not available to others. One way to achieve this is to increase the levels of inorganic ions in the body fluids. In most teleosts, sodium chloride is responsible for 80-90% of the body fluid osmolality, with potassium and calcium ions, urea, and free amino acids accounting for much of the remainder. When temperate marine teleosts are exposed to decreasing water temperatures, the concentration of NaCl in their blood serum increases. The concentration of NaCl in the winter flounder (*Pleuronectes americanus*), increases by 18% when it is transferred from 10°C to -1.8°C (DeVries, 1984). In addition to the inorganic ions, many teleost species living at high altitudes have the ability to produce proteins or glycoproteins which are capable of depressing the freezing point due to their specific affinity for ice crystals. These organic solutes can be responsible for up to 50% of the observed freezing point depression (DeVries, 1983) and have therefore been termed biological antifreeze proteins and glycoproteins.

IV. Biological Antifreezes

1. Antifreeze structure and diversity

At present, five main antifreeze protein types have been identified from fish (Table. 1 and Fig.2). Activity of the antifreeze glycoproteins (AFGP) and proteins (AFP) is measured in terms of the thermal hysteresis they produce. Thermal hysteresis is defined as the difference between equilibrium freezing and melting points. The first antifreeze protein to be isolated was the antifreeze glycoprotein (AFGP) found in the blood of an Antarctic nototheniid *Trematomus borchgrevinki* from McMurdo sound (DeVries and Wohlschlag, 1969). This glycoprotein was found to consist of eight components with molecular weights varying from approximately 33000 Daltons (AFGP 1) to 2500 Daltons (AFGP 8).

All the AFGPs isolated to date are similar in structure. They all have a primary structure in which a repeating tripeptide unit of alanine-alanine-threonine is glycosidically linked to galactose-N-acetylgalactosamine. The frost fish (*Microgadus tomcod*) and the saffron cod (*Eleginus gracilis*) are the only species identified to date that produce antifreeze glycoproteins containing arginine in addition to alanine, threonine, and proline (Fletcher *et al.*, 1982).

The secondary structure of the AFGP has been proposed to be a threefold left handed helix with the hydrophobic surfaces of the disaccharide side chains wrapped closely against the helical backbone (Schrag *et al.*, 1987, Feeney *et al.*, 1994). AFGPs have been

Table 1. Structural characteristics of the five known fish antifreeze types.

Antifreeze Type	Primary Structure	Secondary Structure	Size (kDa)
Type I AFP	repeating sequences of 11 amino acids containing 60 mol% alanine	amphiphillic α-helix	3.3-4.5
Type II AFP	cystine-rich, disulphide linked	two α-helices, 9 β-strands	11-24
Type III AFP	neither alanine nor cystine-rich	β-strands, 1 α-helix	6.5
Type IV AFP	108 amino acids, 17% glutamine	high α-helical content	12.3
AFGP	repeats of Ala-Ala-Thr-disaccharide	three-fold left-handed helix	2.6-33

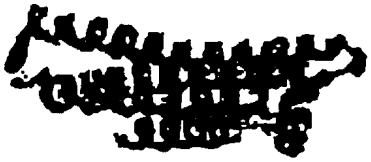
Fig.2. Schematic representation of the five known antifreeze protein types. Adapted from Davies *et al.*, 1999

AFP Type I

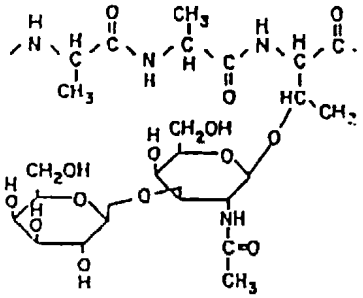
AFP Type II

AFP Type III

AFP Type IV



AFGP



found in the Antarctic nototheniids (*T. borchgrevinki* and *T. hansoni*). the Arctic cods (*Boreogadus saida, Gadus ogac,* and *E. gracilis*), the Atlantic cod (*Gadus morhua*), and the Atlantic tomcod or frost fish (Fletcher *et al.*, 1982, Reisman *et al.*, 1987).

The four known antifreeze polypeptides (AFP) are quite distinct, and differ in their amino acid composition and primary and secondary structure. The first to be isolated and characterized was Type I AFP from the north temperate winter flounder (*Pleuronectes americanus*) (Duman and DeVries, 1975). This type of antifreeze was later isolated from the yellowtail flounder (*Pleuronectes ferrugineous*), shorthorn sculpin (*Myoxocephalus scorpius*), the Alaskan sculpin (*Myoxocephalus verrucosus*), the grubby sculpin (*Myoxocephalus aenaeus*), and the Alaskan plaice (*Pseudopleuronectes quadritabeulates)* (Hew *et al.*, 1980, Reisman *et al.*, 1987).

The Type I AFP is an alanine rich polypeptide. The antifreeze from the winter flounder consists of seven active components. The two major components each have a relative molecular mass of 3300 consisting of 37 amino acids of which alanine accounts for 60 mol % (Fourney *et al.*, 1984). The secondary structure of the Type I AFP has been well researched in the winter flounder. It has been found to be an α-helix and rod-shaped. It is also amphiphilic in that the polar residues (threonine and aspartate) project from one side of the molecule and the nonpolar side chains (leucine and alanine) project down the other side (DeVries and Lin, 1977).

A second polypeptide antifreeze (Type II AFP) has been found in the sea raven (*Hemitripterus americanus*), the smelt (*Osmerus mordax*), and the Atlantic herring

(*Clupea harengus harengus*) (Slaughter and Hew, 1981, Ewart and Fletcher., 1990). This antifreeze type, which is homologous to the C-type lectin, has a primary structure that is cystine-rich. The AFPs from herring and smelt are similar to that of the sea raven in terms of the amino acid composition and presence of half-cystine residues. The AFP of smelt differs in that it contains a small amount of carbohydrate, however, it is unlikely that this carbohydrate is of any great functional significance (Ewart and Fletcher, 1990). Type II AFPs are globular proteins containing five disulfide bridges with a secondary structure consisting of two helices, and nine β-strands in two (Sönnichsen *et al.*, 1995, Gronwald *et al.*, 1998).

The third type of AFP is found in the Arctic and Antarctic zoarcids (*Austrolycicthys brachycephalus, Rhigophilia dearborni,* and *Lycodes polaris*), and the Atlantic ocean pout (*Macrozoarces americanus*). These antifreezes are, like Type I and Type II AFP, multiple polypeptides. The ocean pout AFP consists of 11 active components (Hew and Fletcher, 1985) and the Antarctic eel pout has two predominant components (Cheng and DeVries, 1989). Type III AFP is a rigid globular protein that is neither alanine nor cystine rich. The secondary structure of Type III AFP consists of a number of β-strands and one α-helix, with a flat amphipathic ice-binding site (Jia *et al.*, 1996).

More recently a fourth AFP (Type IV AFP) has been identified from the longhorn sculpin (*Myoxocephalus octodecimspinosis*). It is similar to Type I AFPs from other sculpins in that it is highly α-helical, but differs in that it has a much higher molecular weight (12.3 KD) and a high glutamine content (17%) (Deng *et al.*, 1997).

2. Protective Mechanisms of the Antifreeze Proteins and Glycoproteins

a. Freeze Protection - Interaction with Ice Crystals

All antifreeze proteins, regardless of their different structures and compositions, play a fundamental role in freeze resistance. They are able to depress the freezing point of the body fluids of the fish producing them. The effect on the freezing point is non colligative, that is to say, the freezing point is lowered with very little effect on the melting point and osmolality of the body fluids.

Although the exact mechanism of antifreeze activity is not known, several models have been proposed to explain how antifreeze proteins inhibit freezing and the mechanism underlying the thermal hysteresis effect of antifreeze proteins. A widely accepted model for the mechanism of antifreeze activity is the absorption-inhibition model. The development of this model was based on the fact that the presence of small amounts of impurities can prevent crystallization through binding to embryonic ice crystals and preventing their growth (Franks and Morris, 1978).

Glycopeptide antifreezes have carbohydrate moieties containing hydroxyl groups, and it has been shown that modification or loss of these polar carbohydrate groups result in the loss of antifreeze activity. Therefore it has been proposed that these groups are involved in the adsorption process through hydrogen bonding to oxygen atoms in the ice lattice of ice crystals (Raymond and DeVries, 1977). Structure also plays a role in the

adsorption of the AFPs to ice. Type I AFPs are α-helical rod-shaped molecules. The polar amino acids along one side of the helix are separated by five to seven nonpolar residues and the distance between them is approximately 4.5 Å. Therefore hydrogen bonding between the polar groups and oxygen atoms on the a-axis of the ice lattice is possible through lattice matching (DeVries and Lin, 1977). The ice binding site for Type II AFP has been suggested to reside in the region of the triple-stranded sheet (Sönnichsen *et al.*, 1995). This site corresponds to the calcium binding site, and Type II AFP is dependent on calcium ions for its activity (Ewart *et al.*, 1996, 1998).

Inhibition of crystal growth upon adsorption of the antifreeze molecule has been explained in terms of a kinetic model. In this model it is postulated that adsorption of the antifreeze to ice crystals would block growth in regions covered by the protein and growth would only be possible between the adsorbed molecules. The result would be the formation of curved ice fronts with a large surface area to volume ratio and a high surface free energy. This would inhibit new water molecules from joining the ice lattice and the result would be a decreased freezing temperature (DeVries, 1984). As cooling increases, the kinetic barrier would diminish and ice growth could then occur at the observed freezing temperature (Burcham *et al.*, 1986). Knight *et al.* (1993) have suggested that parts of the antifreeze molecules reside within the ice crystal. This would result in a more permanent binding site since there would be approximately three hydrogen bonds for each polar group on the antifreeze molecule.

b. Hypothermic Protection - Membrane Effects

Rubinsky *et al.* (1990) published findings of an experiment which suggested that antifreeze glycopeptides can protect cold-sensitive cells at hypothermic temperatures (above the freezing point). They found that in the presence of 40 mg/ml AFGP, 83.3% of pig oocytes incubated at 4°C for four hours retained a normal membrane potential across the oolemma, suggesting ion leakage was significantly reduced. At 24 hours, 59-65% of the exposed oocytes had a normal membrane potential. This protective effect was seen at concentrations as low as one mg/ml AFGP. Those oocytes without the AFGP, and incubated at 4°C lost their membrane integrity and died. Therefore it was concluded that the AFGPs interact with the oolemma at hypothermic temperatures and inhibit ion leakage. It was proposed that this protection is the result of the binding of AFGP to available sites on the oolemma, possibly to hydrophilic areas of the membrane proteins. It was later shown that all known antifreezes have the potential to protect mammalian cells, and not only did they minimize hypothermic damage to cells as evidenced by the maintenance of intact membranes, but they also increased cell viability in that low-temperature-exposed bovine oocytes were able to undergo *in vitro* maturation and fertilization (Rubinsky *et al.*, 1991).

Organs intended for transplantation may be stored at cryogenic or hypothermic temperatures. However, problems can arise with both of these methods of preservation. At hypothermic temperatures this is due in part to the reduction of metabolic function to the point where there is insufficient energy produced to maintain normal ion gradients, resulting

in osmotic swelling and cell damage (Hochachka, 1986). Since antifreeze proteins were shown to offer hypothermic protection to cells, it was proposed that perhaps this protection could be extended to a complex organ. Rat liver perfused with 15 mg/ml of Type III AFP from ocean pout and stored at 4°C for 24 hours maintained hepatic cell integrity and function as was shown by the maintenance of bile production. Bile production in controls (no AFP) was considerably lower (more than three times) than in AFP treated liver. This led investigators to propose a role for these proteins in the hypothermic protection of cold-tolerant animals (Lee *et al.*, 1992) and investigations continued into the possible mechanism of the hypothermic protection afforded by AF(G)Ps.

In 1992, patch clamp studies of pig granulosa cells in the presence of Type I AFP from the winter flounder showed that these antifreeze proteins could suppress Ca^{2+} and K^{+} currents (Rubinsky *et al.*, 1992) and later it was shown that Type III AFP from the ocean pout could block Ca^{2+} influx into rabbit parietal cells without affecting Ca^{2+}ATPase (Negulescu *et al.*, 1992). Since the accumulation of intercellular calcium ions leads to cell membrane depolarization, membrane leakage, and cell death, this finding suggested that AF(G)Ps exert their protective action in nonfreezing cold tolerance through a direct interaction with cell membranes, possibly at ion channels.

Recent studies by Tablin *et al.*(1996, 1997) and Hays *et al.* (1996) demonstrated that antifreeze proteins can protect platelets and liposomes, respectively, from cold injury during cooling through the phase transition temperature. Hays (1996)found that leakage of trapped solutes from liposomes was prevented in the presence of AFGPs and Tablin (1996)

demonstrated that AFGP inhibits the cold induced-activation (shape change and secretion of intracellular contents) of platelets during chilling. The AFGPs did not depress phase transition temperature and there was no direct evidence that AFGPs interact directly with the lipid bilayer, however both studies suggest a generalized interaction with membrane phospholipids during phase transitions. These studies taken together with earlier work on the hypothermic protection provided by antifreeze proteins led to the proposal that perhaps antifreeze proteins play a role in the cold acclimation of fish producing them. The observation that some species of fish (yellowtail flounder, American plaice, and adult cod) avoid freezing through habitat selection and still produce antifreeze proteins, although not in amounts sufficient to effectively depress freezing temperature, further suggests such a role for antifreeze proteins.

V. Statements of Research Problems and Goals

Temperature as a limiting factor is very important for ectotherms. Fish have adapted to both high and low temperatures through resistance adaptations of their locomotory and enzyme systems, and the restructuring of their membranes. The antifreeze proteins of north temperate, subpolar, and polar marine teleosts are an adaptation to extreme low temperatures in the presence of ice. Recent evidence points to the possibility that these proteins also have a role in the nonfreezing cold adaptation of the fish that produce them. This led to the present investigation into a possible role for antifreeze proteins in the cold tolerance of fish. Using the

common goldfish (*Carassius auratus)* as a bioassay for the effects of AF(G)Ps on cold tolerance the following questions were addressed:

1) Do antifreeze proteins increase the exposure time necessary to induce cold coma in fish?

2) Do antifreeze proteins increase survival of fish exposed to low temperatures?

Goldfish are ideal animal models for such a study for several reasons. First, goldfish are eurythermal and can withstand exposure to temperatures ranging from zero to forty degrees Celsius depending on their acclimation temperature (Fry, 1968). This allows for experimental manipulation of holding or acclimation temperature. Secondly, at their lower lethal limits, goldfish enter a reversible state of cold narcosis or coma. This allows the investigation into the effects of AF(G)Ps on survival time (time to cold narcosis) and survival rate (number of fish recovering from cold exposure).

This study will provide further insight into the functional significance of antifreeze proteins by testing the hypothesis that in addition to their role in freeze resistance, antifreeze proteins are involved in the process of cold acclimation.

Materials and Methods

I. Experimental animals
1.Holding conditions

Common Goldfish (Carassius auratus. L) 0.5-8 g were obtained from ABCee's Aquatic Imports Lasalle, Quebec in lots of 500 per shipment.. Stock fish were kept in a large tank (48cm x 30cm x 20cm) at the Ocean Sciences Centre, Memorial University of Newfoundland. The tank was continuously supplied with cotton wool-filtered tap water pumped from a nearby pond. Ambient water temperatures ranged from 10-15°C in the spring and summer (April to August), to 5-12°C in the fall and winter (September to March). Stock fish were fed daily with commercial goldfish flakes (Hagen Inc.).

2.Acclimation of experimental animals

Fish used in all cold tolerance experiments were acclimated to 28°C for a period of three weeks prior to experimentation. The change in temperature from the holding temperature to the acclimation temperature was gradual. Temperature was increased by 2°C per day until the acclimation temperature was reached. Temperature in the acclimation tanks was thermostatically controlled to within a degree of the desired temperature. Fish were not fed for 48 hrs prior to experimentation. Fish tested in the winter months were exposed to a long day length photoperiod (16hrs light, 8hrs dark) to mimic summer conditions.

II. Goldfish injection procedure

The goldfish were anaesthetized (2-phenoxyethanol) and injected intraperitoneally with 1 mg/g body weight of either Type I AFP, Type III AFP, or AFGP in 0.7% NaCl (50 µl). Control fish were injected with an equal volume of 0.7% NaCl. Preliminary experiments were carried out to determine whether bovine serum albumin (BSA) could serve as a control protein. However, injection of BSA resulted in the death of all control fish prior to cold exposure. The goldfish were allowed to recover from the anaesthetic (1 hour) at their 28 °C acclimation temperature before being exposed to cold.

III. Blood Sampling

Goldfish were placed on a wet sponge and covered with a moist towel. Blood samples (~ 25 µl) were taken from a caudal blood vessel using 26 gauge needles and immediately transferred to heparinized capillary tubes. The capillary tubes were immediately centrifuged and the blood plasma analysed for antifreeze activity within 24 hours of collection.

IV. Determination of antifreeze activity (thermal hysteresis)

Thermal hysteresis and ice crystal morphology during freezing were used to determine the antifreeze activity of plasma samples taken from injected goldfish. Antifreeze proteins lower the freezing point of a solution without significantly affecting its melting point. Thus a sample containing antifreeze proteins will have a freezing point that is significantly lower than its melting point. The difference between these two values (°C) is termed the sample thermal hysteresis. Freezing and melting points were detected using a Clifton Osmometer (Clifton Technical Physics, Hartford, NY) (Kao et al. 1986). The temperature of the sample at the initiation of freezing and thawing was recorded and the resulting difference between the values converted to degrees Celsius by multiplying by 1.858×10^{-3}.

Antifreeze proteins also alter the morphology of ice crystals. Solutions containing antifreeze proteins form ice crystals that are bipyramidal or spicular instead of the characteristic hexagonal ice shape. The morphology of ice crystals formed within plasma samples also assists in the detection of antifreeze activity. Ice crystal morphology was viewed during freezing and thawing of samples as stated in Kao et al. (1986).

V. Purification of antifreeze proteins

Antifreeze proteins and glycoproteins were purified from plasma obtained from fish caught in Conception Bay, Newfoundland, and held at the Ocean Sciences Centre, Memorial University of Newfoundland.

1. Type I and Type III antifreeze proteins

Purification procedures were similar for both Type I AFP from winter flounder and Type III AFP from ocean pout and followed the method of (Kao *et al.*, 1986). Winter flounder or ocean pout plasma obtained during February and March was applied directly to a Sephadex G75 chromatography column (2.5 x 90 cm). UV spectrophotometry was used to detect proteins in solution. Optical density readings were made at 230 nm . Fractions in the absorbance peak were pooled, lyophilised, and reapplied to the column. Fractions containing antifreeze activity were pooled and lyophilised. Purity of the dried antifreeze protein is estimated to be at least 90% (Kao *et al.*, 1986) with impurities consisting mainly of inorganic ions.

2. Antifreeze glycoprotein

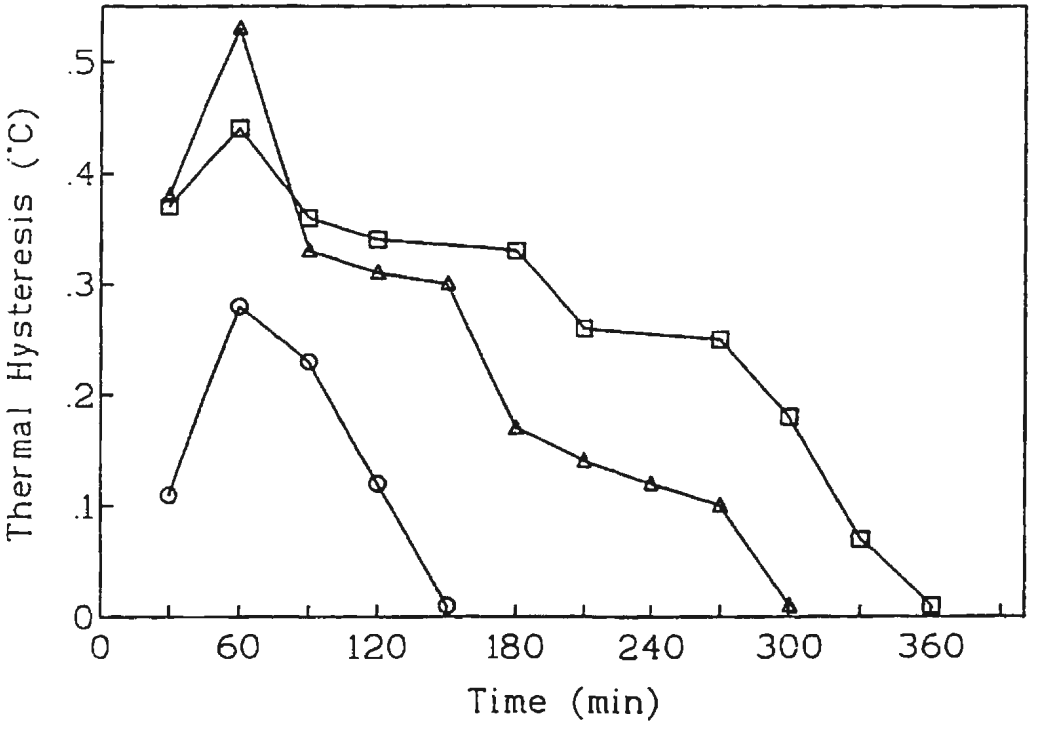
AFGP from Atlantic cod was purified according to the methods of (Kao *et al.*, 1986). Atlantic cod plasma was mixed volume/volume with 20% trichloroacetic acid

(final concentration of 10%) and placed on ice for 5-10 minutes. The sample was then centrifuged at 4°C and 5000 rpm for 10 minutes, and the supernatant was applied directly to a Sephadex G25 chomatography column. The column was eluted with 0.05M NaCl and optical density was read at 230 nm. The protein fractions in the absorbance peak were pooled and lyophilised for reapplication to the column. The second Sephadex G25 column run was eluted with 0.1M NH_4HCO_3. Optical densities of second run fractions were read at 230 nm and samples containing antifreeze activity were pooled and lyophilised and then dialysed against 0.01M NH_4HCO_3 to remove any remaining salts and acid. The dialysed samples were relyophilised and the purity of the resulting product was at least 90% (Kao *et al.*, 1986).

VI. Determination of Antifreeze Clearance Rates

In order to determine the rate of antifreeze protein/glycoprotein clearance from the blood, twelve goldfish were acclimated to 28°C and injected intraperitoneally with one mg/g body weight of either Type I AFP, Type III AFP, or AFGP (four fish per antifreeze type) in 0.7% NaCl (50 µl). Blood samples (10 µl) were taken from each fish once every hour at room temperature and plasma was kept for thermal hysteresis analysis. Samples were staggered so that a sample was taken from two fish per treatment every 30 minutes. Thermal hysteresis was determined with a Clifton Osmometer using the method of Kao et al. (1986). Mean thermal hysteresis values were plotted against time for each antifreeze type (Fig.3). Based on these results, experiments on cold tolerance were carried out when blood antifreeze

Fig.3. Clearance rates of antifreeze proteins from the blood of goldfish at 28 °C. Type I AFP from winter flounder (○), Type III AFP from ocean pout (□), and AFGP from Atlantic cod (△).



levels were highest; approximately one hour after intraperitoneal injection.

VII. Cold Tolerance Experiments

1.Experiment #1:

The effects of Type I AFP on a) time to cold narcosis, b) survival of goldfish subjected to an abrupt drop in temperature

This experiment was carried out during the months of August to November and involved 236 goldfish ranging from 0.3g to 8 g in body weight. The experimental procedure consisted of transferring groups of 4-6 fish, half of which were injected with Type I AFP , to one litre plastic beakers containing fresh water held at a temperature within the range of 0-4.5 °C. Up to five such groups could be exposed to low temperatures in a single day. Fish were marked by fin clips for identification.

Time to cold narcosis was recorded for each fish. Time to narcosis, as stated in Hoar (1955), was the time at which fish no longer responded to mechanical stimulation. Fish were left at its exposure temperature for one hour following cold narcosis at which time they were returned to their acclimation temperature. Goldfish that were alive and swimming actively 24 hours following removal from the cold were recorded as survivors. Blood samples were taken one hour after removal from the cold in order to determine antifreeze activity.

Data for time to cold narcosis was analysed using the SAS program Multivariate

Analysis of Variance (MANOVA) and survival data was analysed with the SAS Logistic Regression and Maximum Likelihood Analysis program using a backward elimination process. (SAS Institute Inc. 1988). In this program the Wald Chi Square statistic tests for the significance of each variable in the model. Only variables meeting the 0.05 significance level for removal from the model were subjected to the maximum likelihood analysis procedure.

2. Experiment #2:

This experiment consists of two parts: a dynamic temperature or tempering experiment, and a static temperature or cold shock experiment. The details of these experiments are described below. The dynamic temperature experiment involved determining the temperature at which 50% of the goldfish in both treated (antifreeze protein-injected) and control (saline-injected) groups become narcotized (NT_{50}). This experiment was a modification of the critical thermal minimum (CTM_{in}) experiments of Bennett and Judd (1992[1]), and served to determine the range of temperatures used for the static temperature experiments. The NT_{50} was used as the midpoint temperature and fish were tested at their NT_{50}, at one degree above NT_{50}, and at one degree below NT_{50}.

a) Dynamic Temperature Experiments - Determination of Temperature to 50% Narcosis (NT_{50})

Goldfish were acclimated to a temperature of 28°C for a period of no less than two weeks prior to experimental manipulation. Fish were fed twice daily with commercial goldfish flakes (Hagen, Inc.) during their acclimation period until 48h prior to experimentation. One hour prior to experimentation, five fish were injected with 1mg/g body weight Type I or Type III AFP, or AFGP in 0.7% NaCl or an equal volume of NaCl (50 µl). All ten fish (saline- and AFP-injected) were then placed in a 4L plastic container partially submerged in an RTE-Series Digital Control Refrigerated Bath/Circulator (Neslab Instruments, Inc.). This procedure was repeated five times to give a total of 25 AFP-treated and 25 controls for each of the experiments. The data was combined for analysis.

A total of six experiments were run, three in the summer and three in the winter, using each of three antifreeze protein types (Type I AFP from winter flounder, Type III AFP from ocean pout, and AFGP from Atlantic cod). Summer experiments were carried out in May (AFGP), June (Type III AFP) and July (Type I AFP). Winter experiments were carried out in October (AFGP), December (Type I AFP), and February (Type III AFP).

The NT_{50} was determined by exposing goldfish to gradually decreasing temperatures until all of the fish enter cold narcosis. The initial temperature of the bath was 28°C and the temperature was decreased at a rate of 2°C x h^{-1}. Temperature was monitored continuously with a Cole-Parmer Digital Thermistor-Thermometer. The fish were checked

at least four times an hour, at which time water temperature and number of fish narcotized were recorded. Cold narcosis was used as the final endpoint, and as in the previous experiment, was defined as the time at which fish exhibited no detectable movements and were unresponsive to tactile stimulation. Fish were removed from the bath following narcosis and blood samples were taken for thermal hysteresis analysis.

For each of the six experiments the cumulative percent narcosis data was probit transformed, and the temperature data was log transformed as described in Finney (1971). The log transformed temperature data was plotted against the probit transformed cumulative percent narcosis data, and the NT_{50} was interpolated from the resulting regression. The NT_{50} data for each experiment (antifreeze-injected and controls) were compared with a Student's t-test. The standard errors of the NT_{50} data were calculated as described in Finney (1971).

b) Static Temperature experiments: The effects of Type I AFP, Type III AFP, and AFGP on the a) time to cold narcosis, b) survival of goldfish after cold narcosis.

As in experiment #1, goldfish were transferred directly from their acclimation temperature to a static or constant temperature for a fixed period of time. Exposure temperatures were determined by dynamic NT_{50} experiments. Goldfish weighing 2-10 g were acclimated to a temperature of 28°C. A total of 48 fish (24 antifreeze-injected, and 24 controls) were used for each experiment. A total of six experiments were run to test the

effects of the three antifreeze protein types used in the dynamic experiments on the cold tolerance of goldfish in summer and winter. Summer experiments were carried out in June (AFGP), July (Type III AFP) and August (Type I AFP). Winter experiments were carried out in November (AFGP), January (Type I AFP), and February (Type III AFP). In each experiment, eight antifreeze-injected fish and eight controls were exposed to three temperatures, at their NT_{50}, one degree above, and one degree below the NT_{50}. The experiments were divided into four runs of two fish/treatment in order to facilitate monitoring of individual fish. As in the previous experiments, fish were marked for identification and AFP treated fish and controls were exposed to test temperatures in the same tank at the same time.

The time to narcosis was recorded for each fish. One hour after narcosis, fish were removed from the test tank and placed in a tank at their acclimation temperature. The total number of fish recovering was recorded for each of the four experimental runs at each temperature. Blood samples were taken one hour after goldfish were returned to their acclimation temperature in order to determine antifreeze activity.

Data for time to cold narcosis and data for survival after cold exposure for each season was analysed using the SAS split plot analysis of variance (Freund *et al.*, 1986). Data for each experiment was combined for the analysis. Temperature (NT_{50}, NT_{50} +1, NT_{50} -1) was the main-plot factor and treatment (AFP-injected and controls) was the sub-plot factor. Since the same experimental procedure was repeated for each antifreeze protein type, type was entered into the analysis as the replication factor. The total data set (summer and winter data) was combined and analysed with a SAS split-split-plot analysis of variance to determine

the effects of season on survival after cold exposure.

Results

I. Experiment #1: The effects of Type I AFP on a) Time to cold narcosis, b) survival of goldfish subjected to an abrupt drop in temperature

A summary of the results of this experiment is given in Table 2. Thermal hysteresis values were recorded for AFP treated fish only since the thermal hysteresis values of saline treated control fish are zero, and in some cases thermal hysteresis values could not be obtained for AFP treated fish due to difficulty getting blood samples from small fish, or because samples were haemolysed and could not be analysed.

a) Time to cold narcosis

Time to cold narcosis was positively related to body weight and exposure temperature ($P < 0.001$) (Table 3). Larger fish remained active, or responsive to touch for longer periods of time than smaller ones. These results are consistent with those reported by Hoar (1955) for goldfish and Pitkow (1960) for guppies. Fish exposed to lower temperatures enter cold narcosis more rapidly than those exposed to higher temperatures (Fig. 4). Treatment with Type I AFP had no effect on the time to cold narcosis.

Table 2. Experiment #1- The effects of Type I AFP on the cold tolerance of goldfish.
Values are expressed as means ± standard error. Numbers in parentheses are sample numbers.

Body Weight		Temp (°C)	Time to Narcosis		Thermal Hysteresis (°C)	Recovery (%)	
saline	AFP		saline	Type I AFP		saline	AFP
1.71±0.16 (3)	1.74±0.06 (3)	3.00	9.28±1.65	17.59±5.77	0.220	0	100
1.06±0.03 (3)	1.07±0.02 (3)	4.00	17.25±6.67	21.90±3.51	0.322	0	100
1.57±0.52 (3)	1.64±0.03 (3)	2.93	10.12±2.25	11.97±4.52	0.197±0.02	0	66.67
0.83±0.90 (3)	1.09±0.04 (3)	2.98	16.98±4.21	22.48±4.37	0.177±0.03	33.33	0
0.81±0.09 (3)	0.86±0.01 (3)	3.36	34.28±4.32	20.08±3.72	0.231±0.02	66.67	33.33
0.75±0.10 (3)	0.86±0.04 (3)	2.99	6.14±1.37	9.94±1.39	0.383±0.07	0	66.67
1.04±0.12 (3)	1.17±0.09 (3)	4.08	15.23±1.21	12.78±2.46	0.254±0.02	0	33.33
1.67±0.11 (3)	1.84±0.15 (3)	2.37	6.57±2.42	7.21±1.41	0.096±0.01	33.33	0
1.84±0.09 (3)	1.97±0.14 (3)	1.23	6.8±0.87	6.03±1.13	0.096±0.01	0	0
1.02±0.12 (3)	1.06±0.04 (3)	1.60	4.06±0.43	4.77±0.84	0.075	0	0
1.39±0.06 (3)	1.71±0.27 (3)	2.3	3.35±0.59	3.56±1.33	0.180±0.06	66.67	33.33
0.90±0.08 (3)	0.96±0.05 (3)	2.23	3.49±0.66	4.45±0.59	0.102	66.67	0
0.76±0.08 (3)	0.89±0.05 (3)	1.29	2.51±0.85	3.04±0.67	0.093	33.33	66.67
0.88±0.16 (3)	0.62±0.05 (3)	1.46	2.21±1.03	3.23±1.03	0.166±0.02	0	33.33
2.07±0.08 (3)	1.7±0.12 (3)	0.61	3.73±0.76	2.9±1.01	0.329±0.02	33.33	66.67
1.17±0.06 (3)	1.08±0.11 (3)	0.65	2.68±0.98	2.38±0.64	0.305±0.06	0	66.67
3.52±0.23 (3)	3.13±0.15 (3)	1.35	5.34±1.49	4.4±0.54	0.124	66.67	100
4.93±0.01 (3)	4.36±0.21 (3)	1.58	6.37±1.46	3.81±0.38	0.093	100	100
5.92±0.25 (3)	1.43±0.06 (3)	1.43	6.6±0.80	7.07±0.37	0.170±0.03	100	100
5.91±0.12 (3)	5.64±0.21 (3)	1.83	5.41±1.22	3.79±0.70	0.103±0.01	100	100

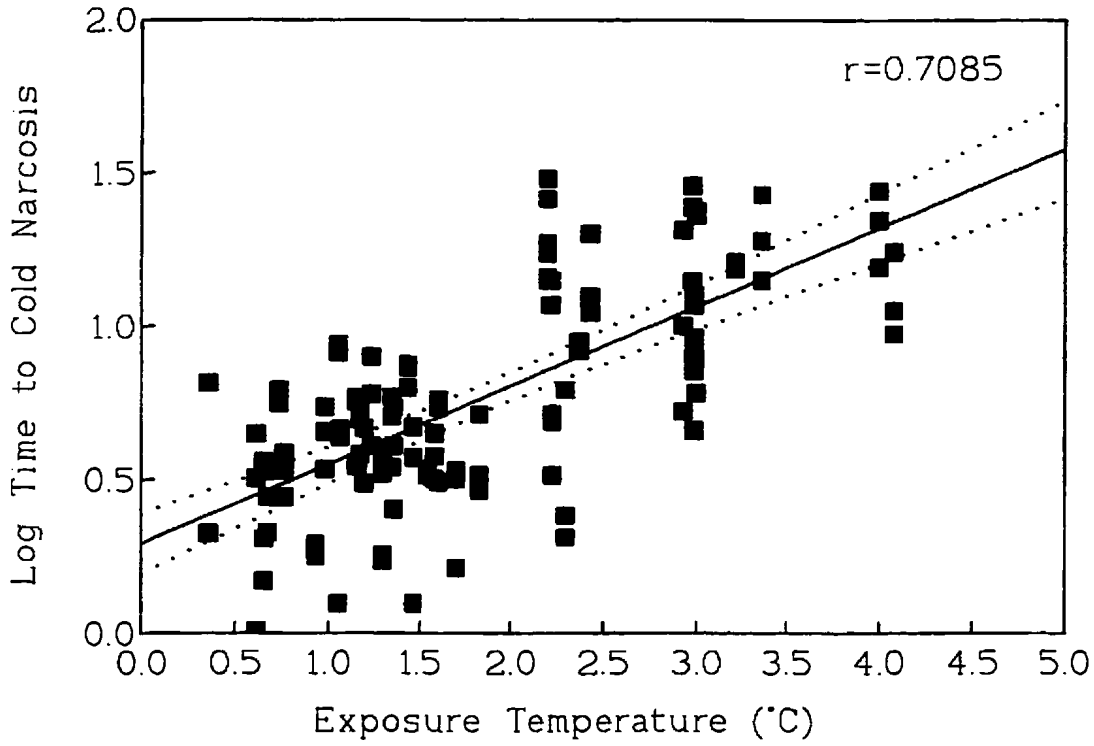
Table 2 Continued. Experiment #1- The effects of Type I AFP on the cold tolerance of goldfish. Values are expressed as means ± standard error. Numbers in parentheses are sample numbers.

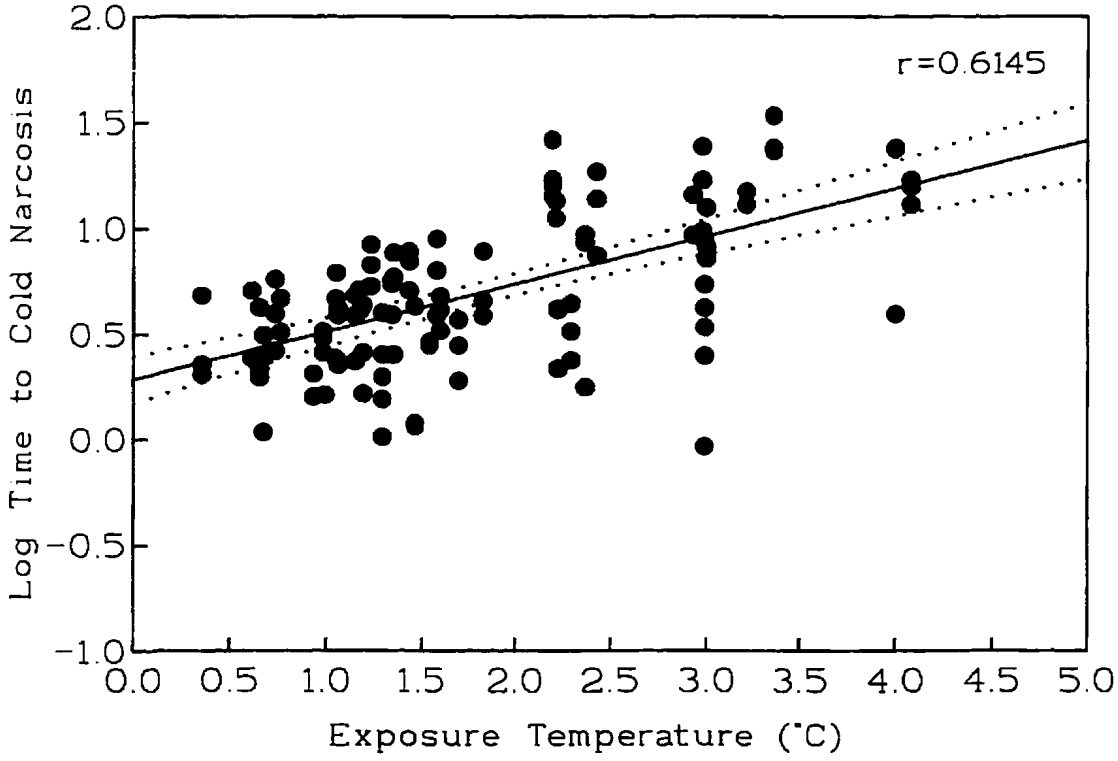
Body Weight		Temp (°C)	Time to Narcosis		Thermal Hysteresis	Recovery (%)	
saline	AFP		saline	Type I AFP		saline	AFP
4.79±0.41 (3)	4.86±0.32 (2)	1.05	4.43±1.10	6.08±2.42	0.223±0.03	66.67	100
5.16±0.16 (3)	4.34±0.16 (3)	0.36	3.03±0.89	4.33±2.20	0.309±0.02	0	33.33
3.87±0.30 (3)	3.66±0.06 (3)	1.17	4.49±0.33	4.93±0.55	0.286±0.01	33.33	33.33
4.71±0.37 (3)	4.58±0.26 (3)	0.98	2.95±0.20	4.47±0.59	0.156±0.02	66.67	100
5.92±0.30 (3)	6.89±0.27 (3)	0.76	4.19±0.48	3.34±0.31	0.194±0.02	33.33	100
3.88±0.26 (3)	3.64±0.06 (3)	0.67	2.22±0.60	2.76±0.36	0.123±0.02	0	66.67
3.40±0.17 (3)	3.22±0.05 (3)	1.34	5.02±0.57	4.83±0.58	0.123	0	0
5.93±0.21 (3)	5.88±0.25 (3)	1.19	2.85±0.71	3.87±0.70	0.177±0.02	33.33	100
5.42±0.28 (3)	5.36±0.08 (3)	0.73	4.10±1.1	5.08±1.05	0.244±0.02	33.33	100
5.81±0.08 (2)	5.36±0.54 (2)	1.54	2.83±0.08	3.33±0.07	0.181±0.01	100	100
5.02±0.20 (2)	5.15±0.10 (2)	1.29	1.77±0.22	2.55±0.75	----------	50	100
5.03±0.03 (3)	5.29±0.26 (3)	1.06	3.78±0.27	4.23±0.28	0.179±0.02	33.33	0
5.59±0.42 (3)	5.63±0.49 (3)	1.15	3.68±0.72	5.01±0.77	0.074±0.01	0	33.33
4.10±0.14 (3)	3.73±0.07 (3)	1.70	2.8±0.52	2.72±0.55	0.184±0.01	66.67	33.33
4.45±0.24 (3)	4.48±0.30 (3)	0.93	1.76±0.15	1.89±0.05	0.197±0.03	33.33	66.67
3.19±0.39 (3)	3.23±0.48 (3)	2.79	2.26±0.72	7.84±2.10	0.175±0.02	33.33	100
6.57±0.31 (2)	6.56±0.34 (2)	3.21	3.98±1.0	5.84±0.44	0.256±0.01	50	100
6.75±0.05 (2)	6.78±0.17 (2)	2.22	2.38±1.16	2.97±1.19	0.181	50	0
4.92±0.12 (3)	4.71±0.06 (3)	2.43	13.23±3.94	14.6±3.42	0.220±0.01	33.33	66.67
6.58±0.40 (3)	6.6±0.03 (3)	2.2	15.6±1.19	16.8±1.5	0.269±0.09	100	100
4.29±0.17 (3)	4.46±0.09 (3)	2.2	18.4±4.86	26.28±2.83	0.315	83.33	100

Table 3 - Experiment #1 - time to cold narcosis. The effects of Type I AFP , temperature. and body weight on the time to cold narcosis of goldfish. Goldfish were abruptly exposed to temperatures ranging from 0-4°C. A Multivariate Analysis of Variance was performed on all the time to narcosis data across all exposure temperatures. N=236 (117 AFP-injected, 119 Control).

Source	Sum of Squares	Mean Square	F Value	Pr>F
Type I AFP	2.373563	2.373563	0.08	0.7765
Temperature	2486.752933	2486.752933	84.62	0.0001
Weight	478.582232	478.582232	16.28	0.0001

Fig.4. The effect of temperature on the time to cold narcosis in experiment # 1. a) Type I AFP-injected goldfish (N=117 , b) saline-injected goldfish (N=119). Dotted lines represent the 95% confidence limits for the regression.


r=0.7085
Log Time to Cold Narcosis
Exposure Temperature (°C)
2.0
1.5
1.0
0.5
0.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0


r=0.6145
Log Time to Cold Narcosis
Exposure Temperature (°C)
2.0
1.5
1.0
0.5
0.0
−0.5
−1.0
0.0 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0

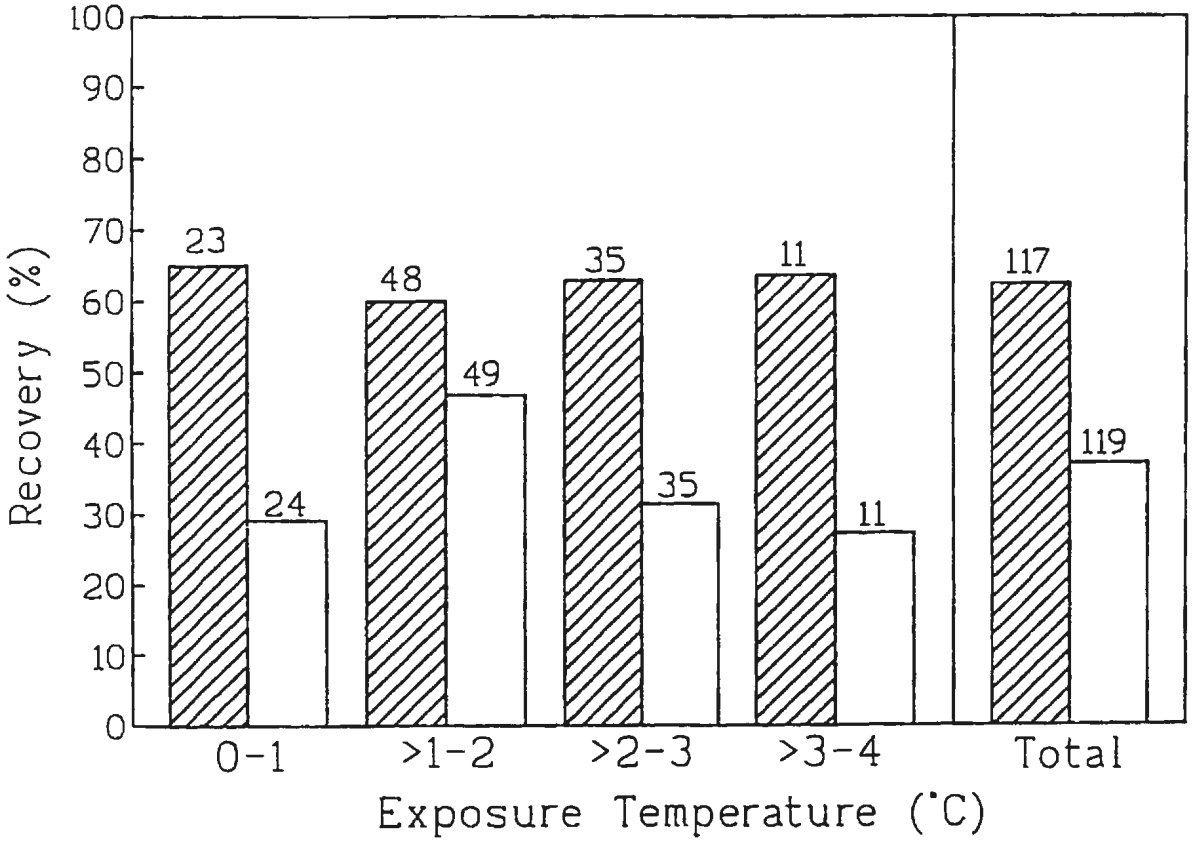
b) Survival of goldfish after an abrupt drop in temperature

Treatment with Type I AFP ($P < 0.0005$), and body weight ($P < 0.0005$) had a positive effect on the survival of goldfish following abrupt exposure to cold temperatures (Table 4). The results indicate that significantly more Type I AFP treated goldfish (62.4%) survived cold exposure than saline treated controls (37%) (Fig. 5) and more large fish survived than small fish. The parameter estimate -1.01 is the increment to log odds of a better outcome (higher % recovery) for small fish. The parameter estimate -0.3767 is the increment to log odds of a better outcome for saline-injected control fish. The odds ratio = 0.433 indicates that control fish are 0.433 times as likely as Type I AFP treated fish to survive cold exposure. The odds ratio = 0.613 indicates that smaller fish are 0.613 times as likely as larger ones to survive cold exposure. Exposure temperature did not influence the number of goldfish surviving cold exposure.

Table 4 - Survival after cold narcosis. The effect of body weight and treatment (Type I AFP) on the survival of goldfish after abrupt exposure to low temperatures ranging from 0-4 °C. N = 236 (117 AFP-injected, 119 controls).

Variable	Parameter Estimate	Standard Error	Wald Chi Square	Pr>Chi Square	Standardized Estimate	Odds Ratio
Weight	-1.0100	0.2829	12.7488	0.0004	-0.278981	0.613
Type I AFP	-0.3767	0.0719	27.4294	0.0001	-0.424663	0.433
Temperature	-----	------	0.6133	0.4335	----------	-------

Fig.5. Recovery rates (%) of goldfish in experiment # 1. The number of fish recovering over a range of temperatures was recorded. ▨ = Type I AFP-injected goldfish. □ = saline-injected goldfish. The numbers above the bars represent the sample number for each temperature range.



II.Experiment #2

a): Dynamic Temperature Experiments: Determination of Temperature to 50% Narcosis (NT_{50})

The results of dynamic NT_{50} determination experiments are shown in Table 5. For each experiment log dynamic temperature was plotted against the probit transformation of cumulative percent narcosis. An example of the plotted data is shown in Fig.6. The NT_{50} (the temperature at which 50% of the fish were narcotized) was interpolated from the regression. A Student's t-test was performed on NT_{50} data (Table 5). There was no significant difference in NT_{50} between treated and control fish for any of the antifreeze types tested, in both summer and winter.

A seasonal comparison of NT_{50} (Table 6) showed a seasonal effect on NT_{50} in experiments with Type I AFP and Type III AFP and their controls, but not with AFGP and its controls. (Table 6). In experiments with Type I and Type III AFP fish tested in winter had a significantly higher NT_{50} than those tested in summer. This suggests that winter fish were less cold tolerant than summer fish in that they become narcotized at significantly higher temperatures. Figure 7 shows a graph of NT_{50} against season. There is a clear seasonal cycle of cold resistance. Resistance to cold increases in the spring and summer, and decreases as the date approaches fall and winter.

AFGP fish were tested in the late spring and late fall which, as figure 7 illustrates, is midway through the seasonal shift in cold resistance. In addition, there seems to be a

Table 5. Experiment #2 (a) -effect of treatment on Dynamic NT_{50}. A Student's t-test was conducted on NT_{50} data.Values are expressed as means ± standard error. For each treatment N = 24.

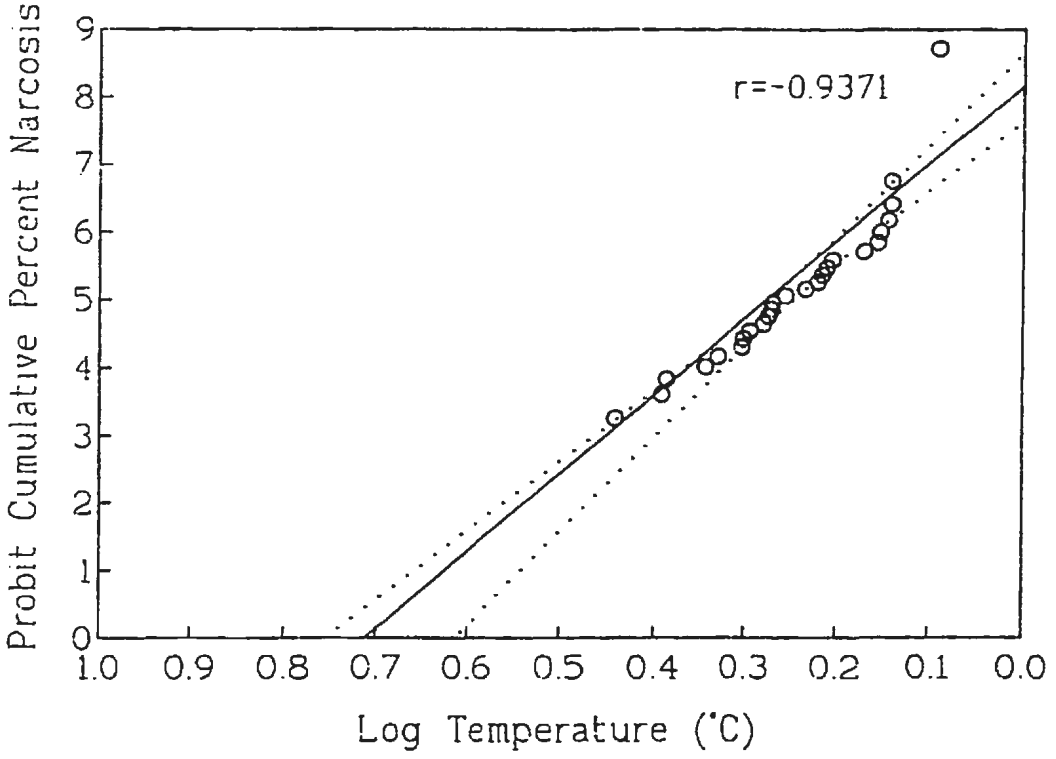
Season	AF(G)P Type	Thermal Hysteresis	Treatment	NT_{50}	t	Pr>|t|
Summer	Type I AFP	0.230 ± 0.13 --------	AFP Saline	2.08 ± 0.13 2.28 ± 0.17	0.935	0.40
Summer	Type III AFP	0.323 ± 0.02 --------	AFP Saline	1.82 ± 0.04 1.93 ± 0.04	1.94	0.10
Summer	AFGP	0.159 ± 0.01 ---------	AFGP Saline	3.69 ± 0.28 3.67 ± 0.14	0.064	1.0
Winter	Type I AFP	0.218 ± .004 ---------	AFP Saline	4.79 ± 0.29 5.06 ± 0.24	0.711	0.50
Winter	Type III AFP	0.274 ± 0.03	AFP Saline	4.26 ±0.22 4.20 ± 0.02	0.206	0.90
Winter	AFGP	0.174 ± 0.01	AFGP Saline	3.04 ± 0.18 3.20 ± 0.17	0.645	0.9

Table 6. Seasonal Comparison of NT_{50} . A Student's t-test was performed on winter and summer NT_{50} data for antifreeze injected fish. For each antifreeze type N=48 (24 AF(G)P treated and 24 controls)

AF(G)P Type	Season	NT_{50}	t	Pr>|t|
Type I AFP	Summer Winter	2.08 ± 0.13 4.79 ± 0.29	8.52	0.001
Type III AFP	Summer Winter	1.82 ± 0.04 4.26 ±0.22	10.89	0.001
AFGP	Summer Winter	3.69 ± 0.28 3.04 ± 0.18	1.96	0.10

Fig.6. Dynamic NT_{50} of goldfish injected with Type III AFP. a) Type III AFP and b) saline. The log dynamic water temperature was plotted against the probit of cumulative percent narcosis, and the NT_{50} interpolated from the regression. Dotted lines represent the 95% confidence limits for the regression. For each treatment N = 25.

a)


r=-0.9371
Probit Cumulative Percent Narcosis
Log Temperature (°C)
0
1
2
3
4
5
6
7
8
9
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

b)

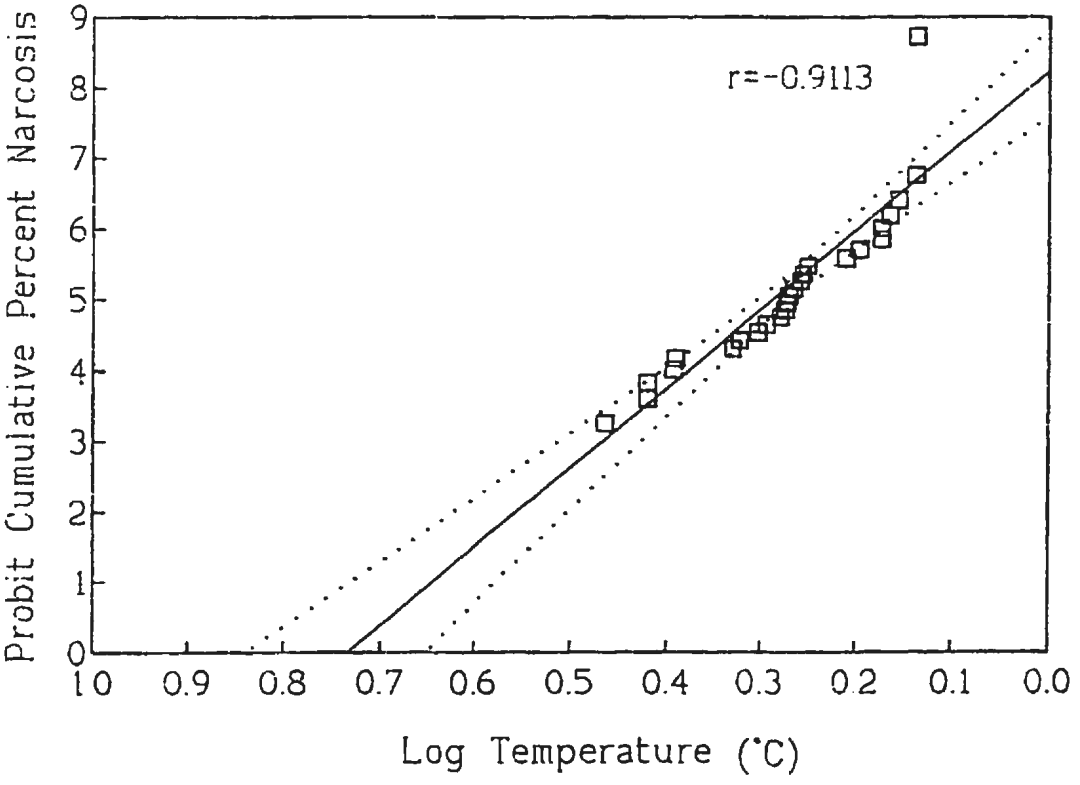
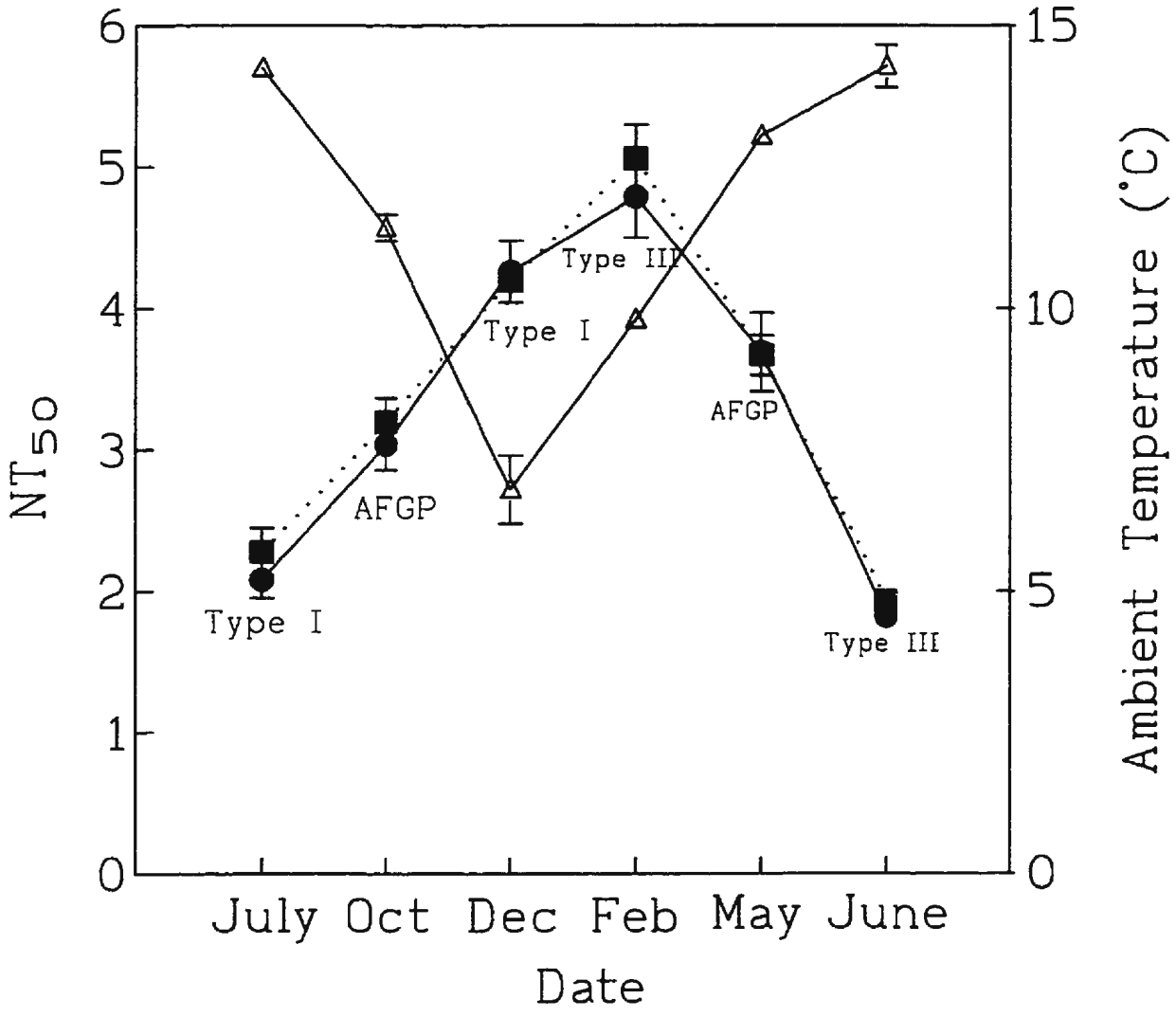

r=-0.9113
Probit Cumulative Percent Narcosis
Log Temperature (°C)
0
1
2
3
4
5
6
7
8
9
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0

Fig.7. Seasonal shift in goldfish resistance to cold. Temperature at 50% narcosis (NT_{50}). and holding temperature (▵) was plotted against the date of experiment for each treatment. ■ = NT_{50} of AF(G)P-injected goldfish. ● = NT_{50} of saline-injected goldfish. AF(G)P = antifreeze protein (AFP) and antifreeze glycoprotein (AFGP).



correlation between holding temperature and NT_{50}. The highest NT_{50} corresponds to the lowest holding temperature, and the lowest NT_{50} to the highest holding temperature. The holding temperature may be affecting the fish in such a way as to make them less cold resistant when they are held at lower temperatures and then acclimated to 28°C. There was only a small difference between the summer and winter holding temperatures of AFGP-treated fish and their controls (13.05 ± 0.05 and 11.43 ± 0.23 respectively) and this may account for the lack of seasonal effect in these experiments.

III. Experiment #2 b): Static Temperature Experiments:

Effects of Type I AFP, Type III AFP, and AFGP on the a) time to cold narcosis,

b) survival of goldfish after cold narcosis

A summary of the results of all the static exposure experiments is given in Tables 7 and 8.

a) Time to cold narcosis -

A SAS split-plot analysis of variance (Table 9) was carried out on all time to narcosis data. AFP type (Type I AFP, Type III AFP, and AFGP) was used as the replication factor in the model design since the fish were tested at each temperature for each antifreeze type. AFP type includes data from both antifreeze protein treated and control fish. For the analysis all temperature data were entered as NT_{50} , NT_{50} + 1, and NT_{50} - 1. Exposure temperature

Table 7. Experiment #2 (b) - summer results . Summary of the results of cold tolerance experiments with Type I AFP, Type III AFP, and AFGP in the Summer. For each exposure temperature and each treatment N=8. A total of 48 fish were used in experiments with each antifreeze type. Values are expressed as means ± standard errors.

AF(G)P Type	Treatment	Thermal Hysteresis (°c)	Weight (g)	Temp (°c)	Time to Narcosis (min)	Number Recovered
Type I	AFP	0.255 ± 0.02	6.486±0.29	3.0	4.79±0.66	7
	Saline	-------	6.685±0.36	3.0	4.46±0.45	6
	AFP	0.242 ± 0.02	6.83±0.47	2.0	3.15±0.31	6
	Saline	-------	7.04±0.43	2.0	2.85±0.48	3
	AFP	0.213 ± 0.04	6.32±0.22	1.0	2.53±0.33	4
	Saline	--------	6.16±0.25	1.0	2.71±0.30	0
Type III	AFP	0.310 ± 0.04	4.79±0.29	3.0	21.37±3.85	6
	Saline	--------	4.9±0.25	3.0	19.28±2.94	6
	AFP	0.228 ± 0.01	4.87±0.38	2.0	3.17±0.57	7
	Saline	---------	4.95±0.31	2.0	2.92±0.74	4
	AFP	0.387 ± 0.03	5.68±0.68	1.0	4.19±0.83	6
	Saline	-------	6.08±0.64	1.0	5.72±0.83	1
AFGP	AFGP	0.187 ± 0.02	6.28±0.46	4.0	19.71±2.12	7
	Saline	--------	6.38±0.53	4.0	16.32±1.4	7
	AFGP	0.198 ± 0.02	7.72±0.76	3.0	12.2±2.78	6
	Saline	--------	8.16±0.61	3.0	9.62±1.8	4
	AFGP	0.187 ± 0.01	6.92±0.5	2.0	5.67±1.15	5
	Saline	------	6.8±0.55	2.0	4.45±0.84	2

Table 8. Experiment #2 (b) - winter results. Summary of cold tolerance experiments with Type I AFP, Type III AFP, and AFGP in the winter. For each exposure temperature and each treatment N=8. A total of 48 fish were used in experiments with each antifreeze type. Values are expressed as means ± standard error.

AF(G)P Type	Treatment	Thermal Hysteresis	Weight (g)	Temp (°C)	Time to Narcosis (min)	Number Recovered
Type I	AFP	0.247 ± 0.02	6.10±0.43	5.0	26.39±4.47	8
	Saline	-------	6.10±0.47	5.0	17.54±3.59	6
	AFP	0.275 ± 0.02	5.43±0.38	4.0	15.28±3.12	4
	Saline	-------	5.57±0.51	4.0	10.49±2.27	2
	AFP	0.261 ± 0.03	3.93±0.23	3.0	4.57±0.95	0
	Saline	--------	3.96±0.24	3.0	4.22±0.75	0
Type III	AFP	0.341 ± 0.07	5.63±0.21	5.0	17.31±2.16	8
	Saline	--------	5.67±0.17	5.0	14.94±1.96	5
	AFP	0.403 ± 0.06	4.81±0.21	4.0	8.36±2.55	4
	Saline	--------	4.8±0.17	4.0	8.2±1.87	2
	AFP	0.266 ± 0.04	5.18±0.34	3.0	4.2±0.52	0
	Saline	--------	4.92±0.30	3.0	3.34±0.38	0
AFGP	AFGP	0.294 ±0.03	6.38±0.36	4.0	30.03±6.38	6
	Saline	--------	6.51±0.45	4.0	27.5±5.9	6
	AFGP	0.286 ± 0.03	7.08±0.43	3.0	14.59±2.52	4
	Saline	--------	6.97±0.44	3.0	17.54±3.91	2
	AFGP	0.222 ± 0.03	7.48±0.91	2.0	5.4±1.08	2
	Saline	---------	8.07±0.94	2.0	4.66±0.99	0

Table 9. Experiment #2 (b) -effect of temperature on the time to cold narcosis : results of a split-plot analysis of variance . N = 48 for experiments with each antifreeze type.

Season	Variable	df	Sum of Squares	Mean Square	F	Pr>F
Summer	AFP Type	2	1581.3987	790.6994	39.90	0.0001
	Temperature	2	3144.5440	1572.2720	6.11	0.0509
	Treatment	1	8.2235	8.2235	0.41	0.5206
	Temp*Treat	2	5.9165	2.9582	0.15	0.8615
	Type*Temp	4	1029.8074	257.4519	12.99	0.0001
Winter	AFP Type	2	1297.5396	648.7698	9.23	0.0002
	Temperature	2	7330.3738	3665.1869	36.99	0.0026
	Treatment	1	185.8990	185.8990	2.64	0.1064
	Temp*Treat	2	132.1202	66.0601	0.94	0.3935
	Type*Temp	4	396.3917	99.0979	1.41	0.2346

($P < 0.05$) had a positive effect on time to narcosis in summer and winter experiments with all three antifreeze types. Fish exposed to higher temperatures (Figs.8, 9), remained responsive for longer periods of time than fish exposed to lower temperatures. There was no treatment (antifreeze) effect on time to cold narcosis in these experiments.

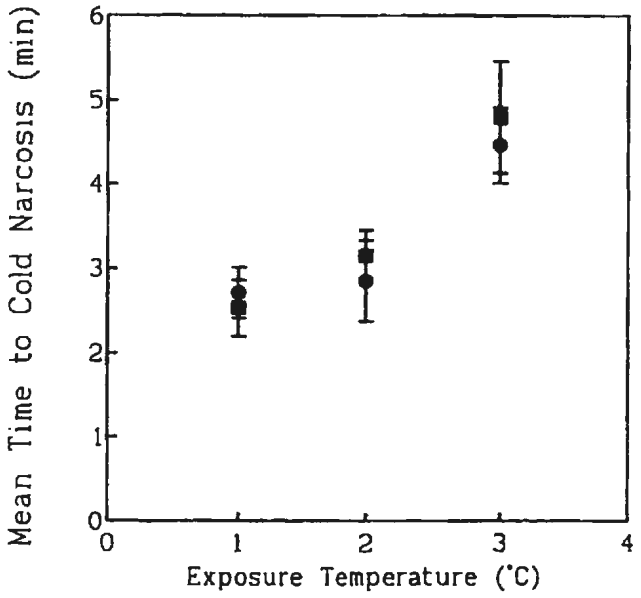
b) Survival after cold Narcosis

For all experiments percent mortality was plotted against exposure temperature (Fig.10). At all exposure temperatures a greater number of fish injected with antifreeze proteins recovered from cold narcosis. A split-plot analysis (Freund et al, 1986) was performed on the survival data. The split-plot procedure analysed the effect of treatment (antifreeze protein/control), temperature (NT_{50}, NT_{50} +1 °C, NT_{50} -1 °C) and antifreeze type (Type I AFP, Type III AFP, and AFGP) on the number of fish recovering from cold exposure. The main plot effect was temperature and the subplot effect was treatment.

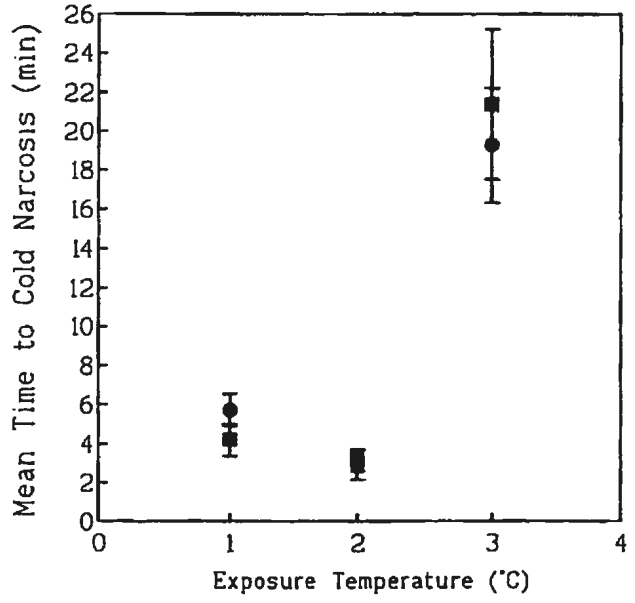
The results of the split-plot analysis of variance are given in Table 10. In both summer and winter experiments, treatment with antifreeze protein had a significant effect on the survival of goldfish after cold exposure. Regardless of antifreeze type, or season, a significantly higher number of fish injected with antifreeze protein recovered from cold exposure compared with controls (Fig. 11). The effect of temperature on recovery from cold narcosis was also highly significant. It is evident from figure 10 that as temperature increased, the number of fish surviving in both treatment and control groups increased. Although there was no significant interaction effect of temperature with treatment, it is evident from the

Fig.8. The effect of temperature on the mean time to cold narcosis of goldfish in experiment #2(b) in the summer. a) Type I AFP, b) Type III AFP, and c) AFGP. ■ = AF(G)P-injected goldfish. ● = saline-injected goldfish. Means ± standard error were plotted for each treatment . N = 8 for each treatment at each exposure temperature.

a)


Mean Time to Cold Narcosis (min)
0
1
2
3
4
5
6
0
1
2
3
4
Exposure Temperature (°C)

b)


Mean Time to Cold Narcosis (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
0
1
2
3
4
Exposure Temperature (°C)

c)

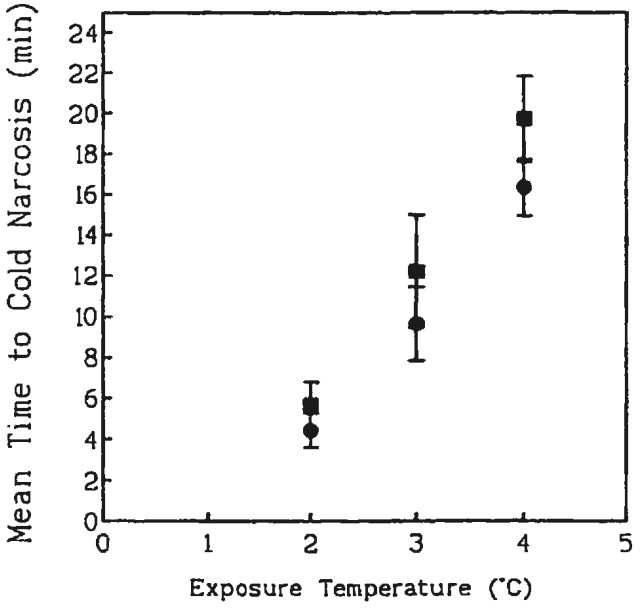
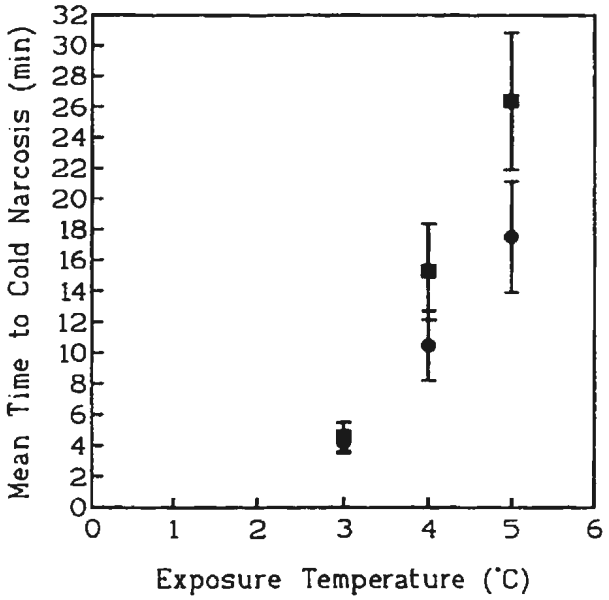
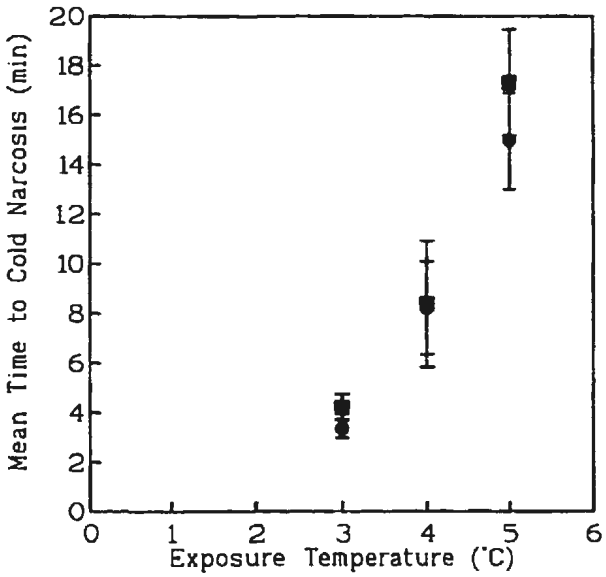

Mean Time to Cold Narcosis (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
1
2
3
4
5
Exposure Temperature (°C)

Fig.9. The effect of temperature on the mean time to cold narcosis of goldfish in experiment #2 (b) in winter. A) Type I AFP, b) Type III AFP, and c) AFGP. ■ =AF(G)P-injected goldfish. ● = saline-injected goldfish. Means ± standard error are plotted for each treatment. N = 8 for each treatment at each exposure temperature.

a)


Mean Time to Cold Narcosis (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
0
1
2
3
4
5
6
Exposure Temperature (°C)

b)


Mean Time to Cold Narcosis (min)
0
2
4
6
8
10
12
14
16
18
20
0
1
2
3
4
5
6
Exposure Temperature (°C)

c)

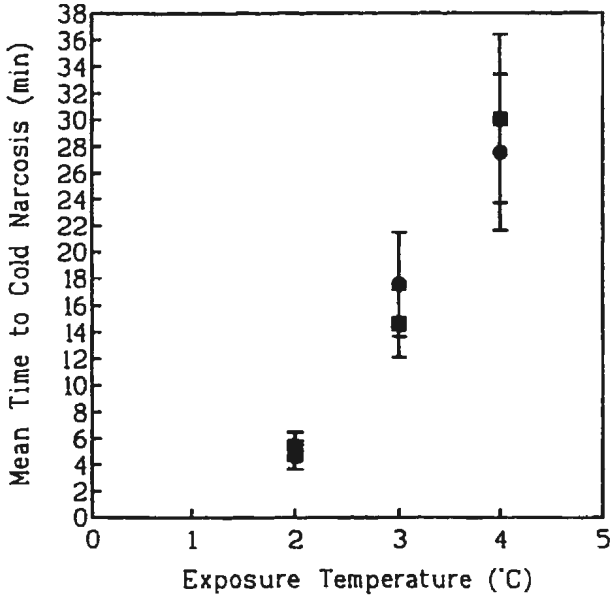
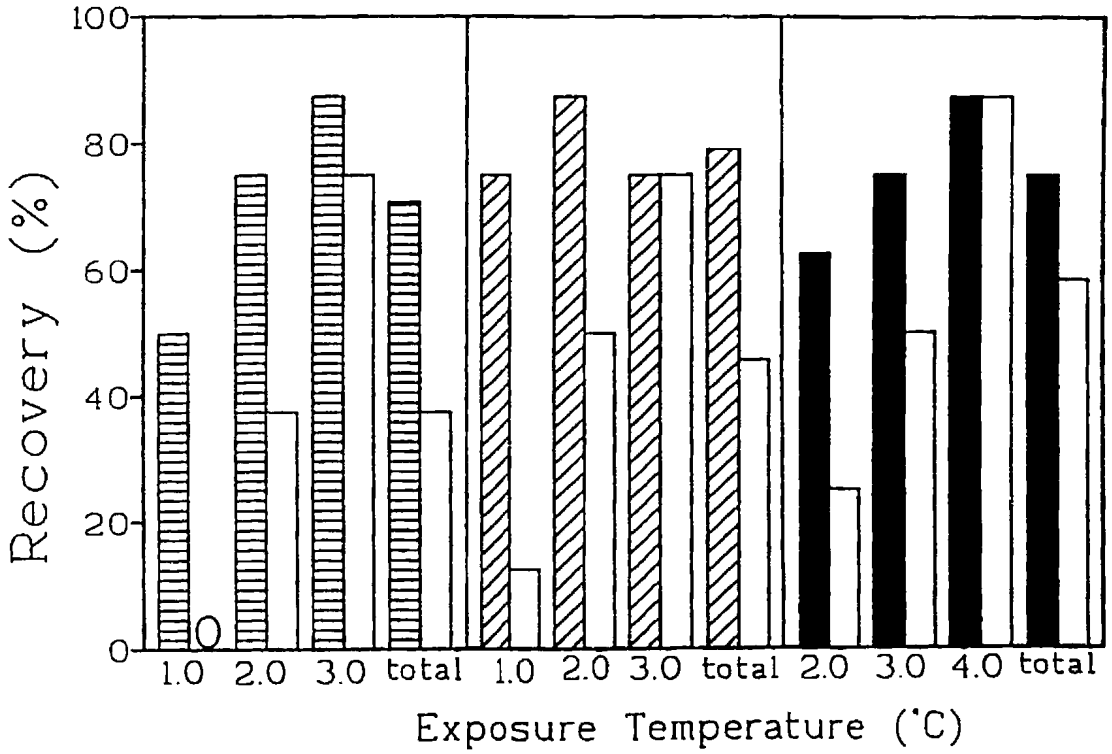

Mean Time to Cold Narcosis (min)
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
0
1
2
3
4
5
Exposure Temperature (°C)

Fig.10. Survival after cold narcosis of goldfish in experiment #2(b) in a) summer and b) winter. Recovery rates (%) were plotted for each exposure temperature. Type I AFP = ▤, Type III AFP = ▨, AFGP = ■, and saline-injected controls = □. N = 8 at each exposure temperature, for each treatment.

a)


100
80
60
40
20
0
Recovery (%)
0
1.0 2.0 3.0 total 1.0 2.0 3.0 total 2.0 3.0 4.0 total
Exposure Temperature (°C)

b)

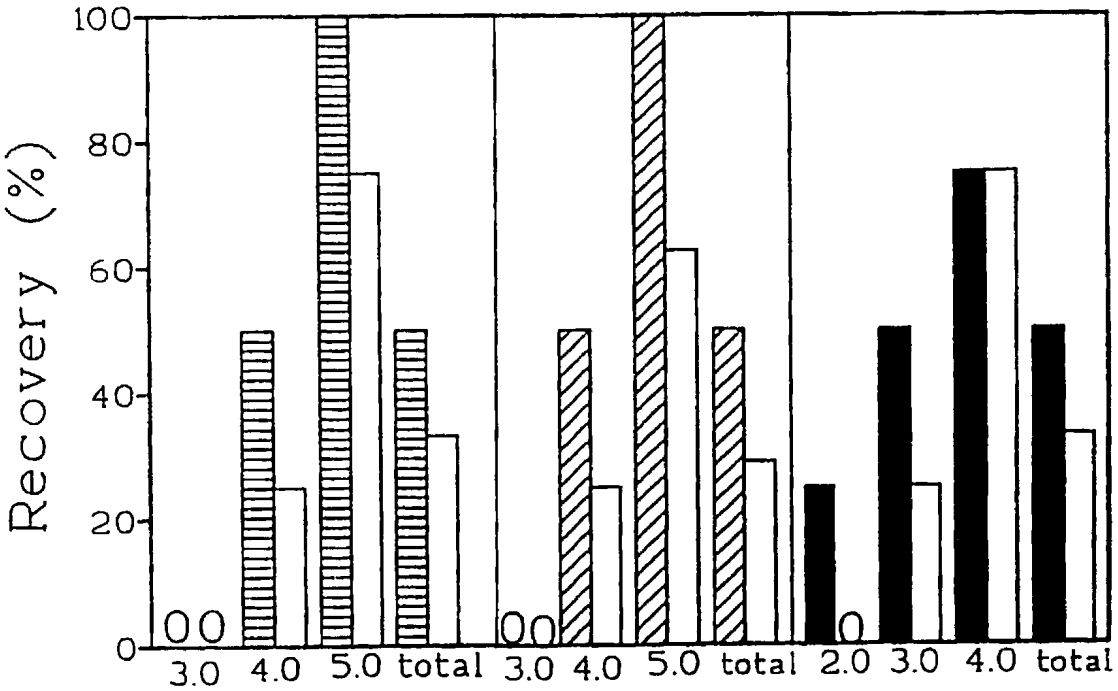

100
80
60
40
20
0
Recovery (%)
0 0
0 0
0
3.0 4.0 5.0 total 3.0 4.0 5.0 total 2.0 3.0 4.0 total

Table 10. Experiment #2(b) - survival after cold narcosis. Split-plot analysis of variance of survival data from static cold exposure experiments with Type I AFP, Type III AFP, and AFGP, in summer and winter. N = 48 for experiments with each antifreeze type.

Season	Variable	df	Sum of Squares	Mean Square	F	Pr>F
Summer	AFP Type	2	2.6333	1.3167	2.10	0.2176
	Temperature	2	113.1333	56.5667	90.27	0.0001
	Treatment	1	11.5694	11.5694	18.46	0.0077
	Temp*Treat	2	0	0	0	1.0000
	Type*Temp	4	0	0	0	1.0000
Winter	AFP Type	2	2.6333	1.3167	2.96	0.1955
	Temperature	2	113.1333	56.5667	127.01	0.0013
	Treatment	1	11.5694	11.5694	25.98	0.0146
	Temp*Treat	2	0	0	0	1.0000
	Type*Temp	4	0	0	0	1.0000

Table 11. Experiment #2 (b) -effect of season on survival after cold narcosis. Split-split-plot analysis of variance of survival data from static cold tolerance experiments with Type I AFP, Type III AFP, and AFGP, in summer and winter. N = 48 for experiments with each antifreeze type.

Variable	df	Sum of Squares	Mean Square	F	Pr>F
AFP Type	2	0.3933	0.1966	0.43	0.6594
Temperature	2	141.3251	70.6626	155.46	0.0001
Treatment	1	33.1668	33.1668	72.97	0.0001
Season	1	29.3857	29.3857	64.65	0.0001
Temp*Treat	2	0	0	0	0
Type*Temp	4	3.0006	0.7502	1.65	0.2308
Treat*season	1	2.9027	2.9027	6.39	0.0281

Fig.11. The effect of antifreeze proteins on recovery of goldfish from cold narcosis. The total recovery rates (%) for all antifreeze protein types tested (■), and all saline controls (□) in summer, and winter.

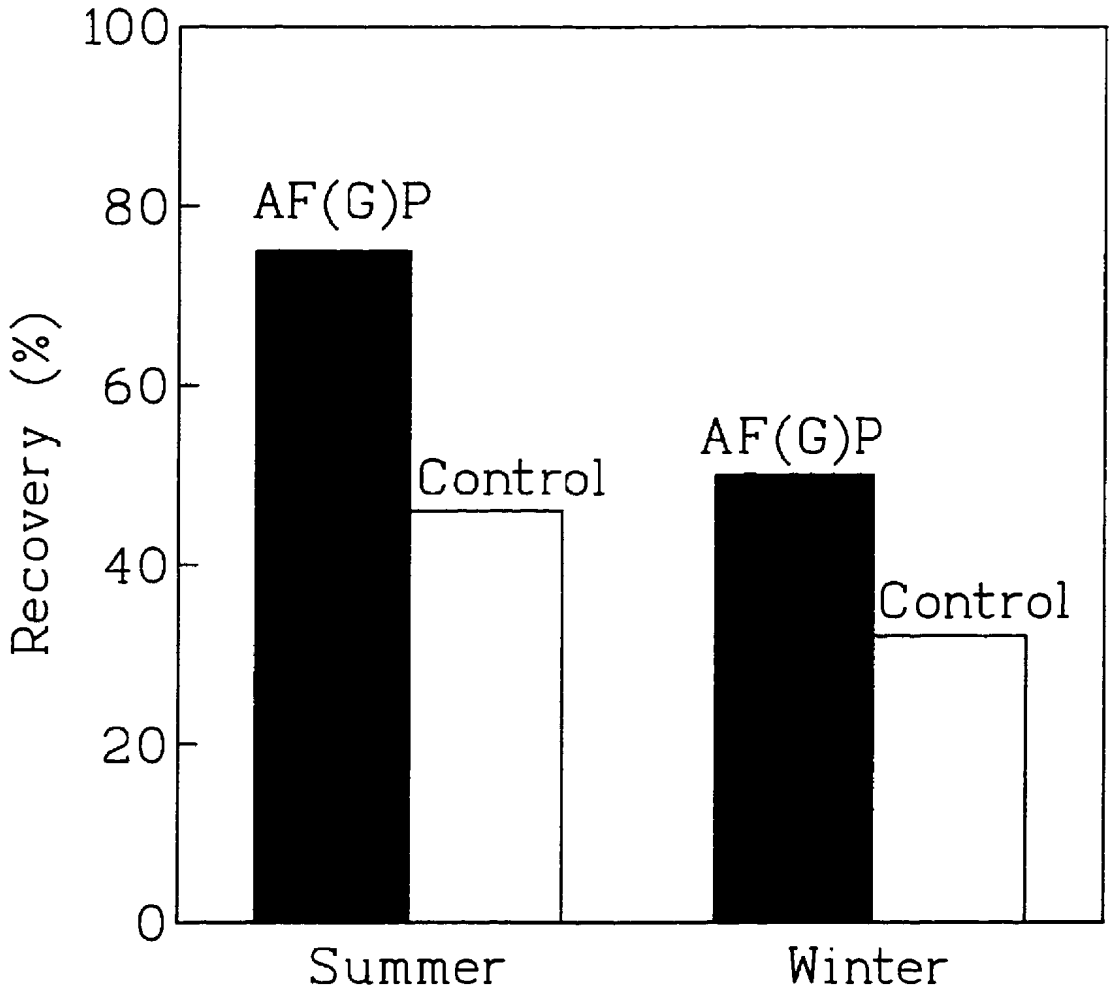


figure that the effect of antifreeze proteins on recovery from cold narcosis was dependent on temperature. In both summer and winter experiments, as temperature increased above, or decreased below the NT_{50}, the positive effect of antifreeze proteins on survival decreased, that is to say, the difference in survival rates between AFP-treated goldfish and controls diminished.

When summer and winter data were compared using a split-split-plot analysis (Table 11), season was shown to have a significant effect on survival. Significantly more fish survived cold exposure in the summer experiments. There was an interaction between season and treatment in the summer experiments. Although significantly more antifreeze-injected fish survived after cold narcosis regardless of season, the difference in survival between treated goldfish and controls depended on season. The difference in survival between treated and control goldfish was less in the winter than in the summer. Clearly there is a temperature at which the goldfish, under the acclimation conditions described for these experiments, are able to survive cold exposure. Likewise, at extreme cold temperatures, the ability of the antifreeze proteins to increase survival is less evident, especially when tolerance to cold is already low, as in winter experiments.

Discussion

I. The Effects of Antifreeze Proteins on the Cold Tolerance of Goldfish

This study provides evidence that fish antifreeze proteins can improve the cold tolerance of goldfish. All of the antifreeze types tested (Type I AFP from winter flounder, Type III AFP from ocean pout, and AFGP from Atlantic cod) seem to offer the same level of hypothermic protection, despite their different primary or secondary structures. This finding offers support to the hypothesis that antifreeze proteins play a role in hypothermic protection, in addition to their well-established role in freeze avoidance.

It is important to note that the intraperitoneal administration of antifreeze proteins did not alter the time to cold narcosis when goldfish were abruptly transferred to low temperatures, nor did they alter the temperature at which the goldfish underwent cold narcosis (NT_{50}) when exposed to a declining thermal regimen. The antifreeze proteins only improved the probability that the goldfish would survive following cold narcosis, and this probability is reduced at extreme low temperatures, especially when natural tolerance is already low, as in the present study's winter experiments.

Two studies have been published on the effects of antifreeze proteins on the cold resistance of fish. Wang et al (1995) produced transgenic goldfish containing an ocean pout antifreeze protein gene (opAFP5), and suggested that the transgenic fish were more cold tolerant than non-transgenics. Unfortunately insufficient experimental details were presented

to allow a scientific evaluation of the validity of their observation.

In another study Wu *et al.* (1998) provided evidence to indicate that the anal administration of Type I AFP improved a) the cold tolerance of juvenile tilapia exposed to an abrupt drop in temperature (26 to 13°C) and b) the cold tolerance of juvenile milkfish exposed to a thermal regimen that declined (26 to 16°C) over several days. Although the validity of these results needs to be verified by further experiments, they do suggest, when taken in conjunction with the results of the present study, that antifreeze proteins can improve the cold tolerance of fish.

The effect of antifreeze proteins in the present study suggests that they somehow prevent or reduce cell or tissue damage arising from what has been referred to as cold shock (Morris, 1987, Drobnis *et al.*, 1993). Cold shock damage has been observed in a variety of cell types, and the injury sustained during rapid cooling is generally irreversible. Survival of cells after cold shock is dependent on continuation of nerve function, maintenance of cellular integrity (ion gradients, cell volume, and protein synthesis), and continuation of cell growth and proliferation (Willis, 1987).

In the present study, when goldfish were abruptly transferred to low temperatures, fish that failed to resume rhythmic ventilation for at least twenty minutes within one hour after removal from the test tank always died. Brett (1952) studied the cold tolerance of young pacific salmon. He concluded that death was probably due to a disturbance of the central nervous system and Pitkow (1960) stated that primary chill coma in guppies results in death due to anoxic damage to a cold-depressed respiratory center. This was partially based on the

observation that unless respiration was permanently reestablished after acute cold exposure. the animal died.

Sun *et al.* (1994) noted the behaviour of tilapia exposed to low temperatures. Initially their activity decreased, possibly due to the effect of cold on metabolism (decreased ATP production) and on muscle enzymes. After some time the fish would lose equilibrium and eventually settle to the bottom of the test tank where they would then cease to ventilate and die. Disequilibrium is an indicator of severe stress in fish (Kindle and Whitmore, 1986) and is a manifestation of some disfunction of the central nervous system (Prosser and Nelson, 1981). The same behavioural sequence in cold-exposed goldfish was observed in the present study suggesting that some disfunction of the central nervous system resulted from both dynamic (gradually decreasing) and static (acute) cold exposure. Since goldfish were able to recover from cold narcosis, this effect is reversible and the presence of antifreeze proteins increases the likelihood of survival after narcosis by reducing damage occurring during the one hour the fish are held at low temperatures after cold narcosis.

The antifreeze proteins may play a role similar to that of trigger factors or cold shock proteins in microorganisms. Trigger factors and cold shock proteins are induced at low temperatures and are essential to cell survival in the cold (Kandror and Goldberg, 1994, Panoff *et al.*, 1998). The functions of cold shock proteins in microorganisms exposed to low temperatures above zero degrees Celsius are related to maintenance of DNA structure and function, or to RNA structure and transcription (Panoff *et al.*, 1998, Schindler *et al.*, 1999). They may also allow protein synthesis and folding to continue at low temperatures,

or help maintain pre existent proteins in a functional form by promoting the refolding of cold-damaged proteins (Kandror and Goldberg, 1994).

It has been suggested that cold shock damage is associated with cell membrane lipid phase transitions and phase separation (Drobnis *et al.*, 1993). During cold shock as individual lipids in the cell membrane reach their phase transition temperature, phase separation occurs, resulting in aggregation of lipids and intrinsic proteins, and leakage of solutes. These phase separations are irreversible. Membranes that are more resistant to cold shock have broad phase transitions, reduced leakage, and reduced phase separation (Drobnis *et al.*, 1993).

Tablin *et al.* (1997) have demonstrated that AFGPs were able to inhibit phase separation of human platelets stored at 4°C up to 14 days. It is possible that antifreeze proteins protect the goldfish in the present study from irreversible cold shock damage by preventing phase separation, particularly in the cells of the central nervous system.

Hochachka (1986) proposed that cold-sensitive cells or organisms could become cold tolerant by preventing the decoupling of active ion pumping and passive ion transport. Rubinsky *et al.* (1992) proposed that the AFPs are able to bind to available sites on the membrane, possibly the hydrophilic parts of membrane proteins and in some way inhibit ion leakage at hypothermic temperatures. It was later proposed that since AFPs could inhibit ion leakage, and suppress Ca^{2+} and K^{+} channels that perhaps the AFPs bind to ion channels (Rubinsky *et al.*, 1990, 1991, 1992, Lee *et al.*, 1992, and Negulescu *et al.*, 1992). Tablin *et al.* (1996) and Hays *et al.* (1996) suggest that AFGP interaction with membranes is a more

generalized interaction with membrane phospholipids since AFGPs prevented membrane leakage at low temperatures but no evidence was presented for a direct interaction of the protein with the membranes of either platelets or liposomes.

A model for AFP binding to membranes has not yet been proposed, but several possibilities present themselves. 1) Hydrogen bonding of antifreeze proteins to membrane proteins. Plasma membranes are approximately 50% protein and transmembrane proteins are amphipathic with the hydrophobic regions passing through the membranes and the hydrophilic regions remaining on the exterior of the lipid bilayer. The AFPs have sites available for hydrogen bonding. Adsorption to ice appears to be mediated largely through hydrogen bond formation. It is possible that binding to the membrane occurs through hydrogen binding of polar groups of the AFPs to hydrophilic groups of the membrane proteins.

The Type II AFPs from the sea raven and smelt have similarities to the C-type or calcium dependent lectins (Ewart *et al.*, 1992). The smelt AFP precursor is homologous to the carbohydrate recognition domains of the C-type lectins. Lectins are carbohydrate-binding proteins that have binding sites that recognize specific sequences of sugar residues of cell-surface glycoproteins, proteoglycans, and glycolipids. The C-type lectins have a calcium binding site. This site is nonfunctional in the sea raven AFP but smelt AFP has a functional binding site. Therefore it may be that the carbohydrate recognition domains of C-type lectins may be able to interact with ice in a manner similar to the AFPs. (Ewart *et al.*, 1992). Likewise the Type II AFPs may have similar binding sites that allow them to bind to glycosylated membrane proteins.

2) Hydrogen bonding with water in and around ion channels. Ion channels or channel proteins form highly selective water-filled pores. These pores allow the passive transport of specific ions. The pores are very small and ions have to shed most of their associated water in order to get through. Through binding with the water in and around the ion channels it may be possible that the AFPs can partially block the channel making it even more difficult for the ions to permeate. When the sodium-potassium pump is inactivated during hypothermia, passive ion leakage occurs at a faster rate than active ion pumping, leading to membrane depolarization, activation of voltage-gated calcium ion channels, and eventual membrane disruption. Partial blocking of the K^+ leak channels would slow down this process and extend the life of cold-exposed organisms.

The results of these experiments suggest that antifreeze proteins can be used to produce fish better able to tolerate cold. Antifreeze proteins could be used to increase the cold tolerance of fish species for cold water aquaculture or could be used to enhance the survival of fish during transport, since they may be exposed to rapidly decreasing temperature.

II. Seasonal Effects

In the present study a seasonal pattern of cold resistance (NT_{50}) and tolerance (survival after cold narcosis) was evident. Fish tested in the winter months had a significantly higher NT_{50} and significantly more fish survived summer experiments than winter experiments. Resistance began to decrease in the fall or early winter (October), and to increase in the late spring or early summer (May). Cold resistance was highest in June and lowest in February. These results suggest that the cold tolerance of the goldfish tested was lower in the winter than in the summer. This is the opposite of what was expected based on previous experiments. Earlier studies have shown that the cold tolerance of fish tends to increase in the winter and with cold acclimation (Hoar, 1955, 1959, Starling *et al.*, 1995).

Hoar and Robertson (1959) observed that short day length exposed goldfish tended to become deeply narcotized and the tendency to show cold narcosis was stated to be a characteristic of naturally produced winter fish. This may offer an explanation of the results of the present dynamic NT_{50} experiments. Goldfish tested in winter may have had a greater tendency toward cold narcosis than those tested in summer and therefore entered narcosis at a higher exposure temperature. It does not explain, however, why fish tested in winter were less likely to survive static temperature experiments than those tested in summer.

One possible cause of the downward shift in cold tolerance in the winter is the acclimation process of winter fish. Changes in acclimation temperature generate seasonal

modifications of cold tolerance in pinfish and many other fish species (Bennett and Judd, 1992[2]). Fish that were tested in the winter were purchased in the winter. The acclimation history of these animals is not known. When they arrived, they were placed in stock tanks at ambient temperature and photoperiod. Test fish were acclimated to a long day photoperiod two months prior to experimentation and a temperature of 28°C at least three weeks prior to experimentation. Acclimation to a higher temperature would involve changes in membrane structure (decreased unsaturation of lipid bilayer) and enzyme systems. When the animals were then tested at cold temperatures, they likely attempted to restructure their membranes to compensate for the temperature change. The switching from cold, to warm, to cold temperatures may have had a detrimental effect on the physiology of the animal. To remedy this problem in future experiments of this nature, winter fish should be kept at a temperature closer to summer ambient temperatures and then acclimated to the desired temperature.

Conclusions and Perspectives

This study demonstrates that antifreeze proteins are able to increase the survival of cold-exposed goldfish. It may be that the antifreeze slows the effects of cold on osmoregulatory processes or operates on the membranes of the nervous system to prevent the damage to nervous cell membranes that eventually lead to nervous system failure and death.

The exact cause of death in cold-exposed goldfish is not known. Future experiments investigating the cold on whole organisms and cell cultures will provide more insight into the problem. Future cold tolerance experiments should investigate the effects of AFPs on tissue water content/distribution, ion concentration, and haematocrit in order to determine if antifreeze proteins can diminish the effects of cold on osmoregulation. The effects of AFPs on ion leakage can be investigated by using patch clamping on cultured fish cells such as a fin cell culture.

The discovery of a suitable protein control will greatly improve cold tolerance experiments using antifreeze proteins. Mutant antifreeze proteins produced by site-directed mutagenesis may provide such a control. These mutant AFPs are altered in such a way that antifreeze activity (adsorption to ice and non-colligative lowering of freezing point) is inhibited. The use of such a control would test whether hypothermic protection is a function unique to antifreeze proteins and it could lend insight into the mechanism of antifreeze binding to membranes as well as to ice by determining whether specific ice binding sites on the

AF(G)Ps are also involved in binding to membranes.

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